ISOLATION AND CHARACTERIZATION OF A SPINAL CORD PROTEIN

by

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ABSTRACT

A highly immunogenic spinal cord protein (SCP) was extracted from bovine spinal cord with 0.1 M sodium chloride. SCP was detected only in bovine nervous tissues. It was also detected in human, rabbit, rat, and mouse spinal cord extracts.

Immunoelectrophoretic analyses revealed that bovine SCP occurred in three molecular forms. Two forms are large enough to be retained by dialysis tubing and have the electrophoretic mobilities of a β_1-globulin (β_1-SCP) and a γ-globulin (γ-SCP) respectively. The dialysable form of the antigen has an electrophoretic mobility of a γ-globulin.

β_1-SCP was isolated by batch absorption of bovine cord extracts with DEAE-Sephadex A-50, followed by chromatography on CM-52 cellulose. γ-SCP was purified by chromatography of bovine spinal cord extracts on CM-Sephadex C-25.

Pretreatment of guinea pigs with purified β_1-SCP or γ-SCP prevented them from developing experimental allergic encephalomyelitis when they were challenged with bovine spinal cord or bovine encephalitogenic protein. The protective activity of the SCP is assumed to be mediated by anti-SCP immunoglobulin.

RÉSUMÉ

ISOLATION ET CARACTÉRISATION D'UNE PROTÉINE DE LA MOELLE ÉPINIÈRE

Une protéine (SCP) de grand pouvoir immunogène a été extraite, avec NaCl 0.1M, de la moelle épinière de boeuf, protéine qui a été localisée uniquement dans le tissu nerveux.

L'analyse immuno-électrophorétique démontra trois formes moléculaires de SCP, dont deux furent retenues à l'intérieur du sac à dialyse. Ces deux formes ont démontré des mobilités électrophorétiques d'une β_1-globuline (β_1-SCP) et d'une γ-globuline (γ-SCP) tandis que la forme dialysable la mobilité d'une γ-globuline.

β_1-SCP a été isolé par absorption en volume des extraits de la moelle épinière de boeuf avec DEAE-Sephadex A-50, suivie par chromatographie sur cellulose CM-52, γ-SCP a été isolé par chromatographie des extraits sur CM-Sephadex C-25.

Des cochons d'Inde prealablement traités avec β_1-SCP ou γ-SCP purifiés n'ont pas développé d'allergie encéphalomyélite après l'injection de moelle épinière our de protéine encéphalitogénique. L'immunoglobuline anti-SCP serait responsable du pouvoir protecteur de SCP.

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LIST OF ABBREVIATIONS

BSC	bovine spinal cord
SCP	spinal cord protein
BEP	bovine encephalitogenic protein
anti-D-BSCE serum	antiserum raised with a bovine spinal cord extract purified by absorption with DEAE-Sephadex A-50
EAE	experimental allergic encephalomyelitis
CNS	central nervous system
CFA	complete Freund's adjuvant
IFA	incomplete Freund's adjuvant
DTF	dialysis tubing filtrate
PHA	passive haemagglutination
CM-cellulose	carboxymethyl-cellulose
DEAE-Sephadex	diethylaminoethyl-Sephadex
M	molar
g	gram (s)
Tris	tris (hydroxymethyl) aminomethane
µg	microgram
µl	microlitre
BDB	bis-diazotized benzidine

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I. INTRODUCTION

In recent years, there has been an increase in the number of studies of the proteins of the central nervous system. It is estimated that several hundred different proteins exist in saline extracts of brain (1,2). It is believed that these proteins take part in the specific and complex activities of the brain such as interneuronal transmission, perception, memory and learning.

PROTEINS OF THE SOLUBLE EXTRACT OF NERVOUS TISSUE

Lajtha (3) showed recently that 30 to 50% of the brain proteins may be extracted with aqueous media. Electrophoretic methods have been used to estimate the number of proteins in aqueous extracts of brain. Thus, MacPherson and Liakopoulou (4), using immunoelectrophoretic analyses found that aqueous extracts of rat brain contained at least 15 antigenic proteins. Similarly, Hatcher and MacPherson (5) found the same number of protein components in the soluble fraction of bovine brain. Vos and Van der Helm (6) separated rabbit brain extracts into 30 components by electrophoresis in polyacrylamide gel. Bailey and Heald (7) separated proteins in human brain extracts into 10 components by starch gel electrophoresis. Many of the immunogenic proteins are shared with other organs of the same species and relatively few of them are restricted specificly to nervous tissue.

1. S-100 Protein

This highly acidic protein was discovered by Moore and McGregor (2). It is so named because of its solubility in saturated ammonium sulfate solution. S-100 is present in higher concentration in cerebral white matter than in the cerebral cortex (2). It has a molecular weight of 21,300, contains three subunits of molecular weights 7,000 (8) and a high content of glutamic and aspartic acids. Because of its specific interaction with calcium, it may play a role in binding calcium in the brain and regulating its availability.

2. α-BOSAB

This protein was isolated from bovine brain extracts by Hatcher and MacPherson (5). It has the electrophoretic mobility of an α-globulin, and was named α-brain organ-specific antigen from the bovine (α-BOSAB). It was estimated to have a molecular weight of 84,000 by gel filtration and its amino acid analysis showed that it contained 20% of glutamic and aspartic acids (9). α-BOSAB was found to be a component of glial cells. It appears earlier in development than the main SRANT antigen or the S-100 protein, for it can be detected in bovine fetal brain as early as the seventh month of gestation and in human fetal brain as early as the third month of gestation. Its function has not been determined so far.

3. α-Antigen

This protein was purified from rat brain extracts by Bennett and Edelman (10).

The antigen was also present in extracts of rat spinal cord and peripheral nerve. The protein occurred in various aggregates stabilized by disulfide bonds. It was found to consist of a single type of subunit having a molecular weight of about 39,000. It is mildly acidic and is immunologically unrelated to S-100 protein.

4. SRANT Protein

This organ antigen was isolated by MacPherson and Liakopoulou (4) and was named species-restricted antigen of nervous tissue (SRANT) because it was found only in aqueous extracts of rat nervous tissue. The main SRANT protein occurs in the adult rat in two molecular forms that have the electrophoretic mobilities of an α_1-globulin and an albumin. The molecular weight of the SRANT protein was estimated by gel filtration to be 70,000 (11). SRANT was detected in the cerebral cortex, hippocampus, cerebellum, spinal cord and sciatic nerve extracts. It is barely detectable at birth but becomes one of the major antigens of the soluble fraction of rat brain after the first week of life.

5. Neurotubular (Microtubular) Protein

Neurotubules have a diameter of 240 A° and are made up of linear arrays of the subunits of neurotubular protein (12). Disruption of the neurotubular structure is caused by the binding of colchicine to the protein subunits of the neurotubule. The neurotubule protein subunit is phosphorylated on a serine residue in the presence of

brain protein kinase and adenosine triphosphate (ATP). Adenosine 3',5'-monophosphate (cAMP) stimulates this phosphorylation reaction (13). In the developing chick brain, 10-20% of the soluble protein is neurotubule protein while in the adult chick brain, only 2 to 2.5% of the soluble protein (14) is neurotubular protein.

6. Neurofilament Protein

The neurofilament has a diameter of 80-100 A° and is present in axons and dendrites and appears to be an essential part of the cytoskeleton (15). Its protein subunits can be disaggregated with detergents, or 6M guanidine hydrochloride (16, 17). The major protein subunits have molecular weights of 45,000 and are acidic. These proteins are involved in growth and maintenance of neurites.

PROTEINS PECULIAR TO CEREBROSPINAL FLUID (CSF)

In 1961, MacPherson and Cosgrove (18) and Clausen (19) discovered two globulins in human cerebrospinal fluid (CSF) not readily detectable in normal human serum. Immunoelectrophoretic analyses showed that the two proteins have the electrophoretic mobilities of a γ- and a β-globulin respectively. The protein with the electrophoretic mobility of a γ-globulin was named γ_c-globulin by MacPherson and was found to comprise 5% of the proteins of normal CSF. Later, the presence of the two proteins in human CSF was confirmed by Hochwald and Thorbecke (20) and by Laterre and Heremans (21). MacPherson used quantitative immunochemical methods and found that the

γ_c-globulin concentration in CSF drawn from patients with demyelinating diseases contained, on the average, 30% less than the amount found in CSF specimens withdrawn from normal subjects (22).

MacPherson and Saffran (23) discovered a comparable γ_c-globulin in bovine CSF. This γ_c-globulin also existed in two molecular forms with different electrophoretic mobilities. The protein also occurs in two different sizes with one form being small enough to pass through dialysis tubing. The molecular weights of the large and small γ_c-globulin were estimated by gel filtration to be 30,000 and 10,000 respectively.

THE MYELIN SHEATH

The myelin sheath is currently believed to be a modified plasma membrane which is wrapped around the nerve axon in a spiral fashion (24). The actual mechanism of myelin formation is still not completely clear. During the early stages of formation of the myelin sheath, the membrane is wound loosely around the axon; then compaction begins along with the synthesis of basic proteins and cerebroside (25). The main function of the myelin sheath is to facilitate conduction in axons. Myelin is composed of 70-80% lipid, 20-30% protein and is characterized by a low water content. It contains at least three major types of protein components: 1) the Folch-Pi-Lees proteolipids; 2) the encephalitogenic basic protein; and 3) the Wolfgram type proteins.

PROTEINS OF MYELIN

1. The Folch-Pi-Lees Proteolipid

The name proteolipid was given by Folch-Pi to the protein-lipid material that could be extracted from brain tissue with chloroform-methanol, but was insoluble in water (26). It is attacked by pronase, but is resistant to trypsin and pepsin. Its molecular weight has been reported to be 34,000.

2. Encephalitogenic Basic Protein

The great interest in the basic protein of myelin is due to its encephalitogenic property. As far as is known, it is the only brain protein which, when injected into experimental animals, induces a disease called experimental allergic encephalomyelitis (EAE). This disease is characterized by loss of weight, incontinence, ataxia and, in severe cases, by paralysis of the hind legs. Histological lesions are found in the brain and cord. The assumption that myelin was the encephalitogenic constituent of nervous tissue was based on Morgan's observation (27) that white matter was more encephalitogenic than gray matter, and, that neonatal tissue, devoid of myelin, was inactive (28). This assumption was strengthened subsequently when it was reported that white matter proteolipids were encephalitogenic (29,30,31). A collagen-like water-soluble protein extracted by Roboz et al. (32) from defatted spinal cord was also found to produce severe EAE in guinea pigs (33). Finally, the basic protein, isolated from purified myelin by Kies, Alvord and their collaborators (34,35,36,37) was shown to be the encephalitogen. These workers used 0.01 N HCl to extract basic protein from defatted brain or spinal

cord. Isolation of the purified basic protein from defatted nervous tissue can be achieved by a variety of chromatogrc hic procedures (38,39,40,41,42), or by a single step chromatography on sulfoethyl Sephadex (43). The yield of protein is about 1% of the original dry weight of the starting material; 1-2 μg emulsified with complete Freund's adjuvant induced EAE in approximately 50% of guinea pigs.

The biological activity of the basic protein is undiminished even after it has been heated at extreme pH values (44), or autoclaved at over 100°C (45). Optical rotatory disperson measurements showed that it exists in a disordered randomly-coiled configuration (46). The molecular weight of the basic protein determined by a variety of methods is in the range of 16,000-18,000 (42,46,47). The isoelectric point of the basic protein is pH 10.6, and it contains about 25% of basic amino acids. The basic proteins of myelin from all mammals except the rat appear to be very similar in size, and amino acid composition. The rat contains two encephalitogenic components differing in molecular weight (48,49). The larger protein is similar in size, amino acid composition, and encephalitogenic activity to the basic protein found in other mammals while the smaller protein has a different amino acid composition and is less active biologically (48). The complete amino acid sequences of the bovine and human basic proteins have been reported by Eylar (50) and Carnegie (51).

Eylar and Hashim (52) found that digestion of the bovine basic protein with pepsin yielded an active 14-amino acid encephalitogenic peptide which contained tryptophan and was active in guinea pigs. After tryptic digestion, a smaller

9-amino-acid polypeptide was released. It has the following sequence:-

Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys

The essential requirements for disease induction are the three residues of tryptophan, glutamine and lysine. The other residues could be replaced without significantly altering the encephalitogenic activity. This peptide of bovine origin is encephalitogenic in rabbits, guinea pigs but not in monkeys. Kibler et al. (53) obtained a peptide containing 45 amino acids, but no tryptophan, by digesting bovine basic protein with pepsin. A similar peptide has also been isolated by Eylar et al. (54). This peptide is encephalitogenic in rabbits but not in guinea pigs (55). This suggests that the myelin basic protein contains more than one encephalitogenic site and that different species exhibit diverse responses to the active regions of the basic protein.

Eylar and Hashim (56) reported that the encephalitogenic basic protein lost its biological activity after treatment with 2-hydroxy-5-nitrobenzyl bromide which blocked the single tryptophan residue. Other workers (57,58) confirmed this observation. Eylar et al. (59) had synthesized a peptide by the Merrifield method with sequence resembling those around the tryptophan residue of basic protein. It is highly encephalitogenic and as low as 0.33 μg will induce histological lesions and clinical signs in the guinea pig.

3. Wolfgram Proteolipid

This protein was first described by Wolfgram (60) and has since been known as Wolfgram protein. The protein was extracted from myelin with acidified chloroform-methanol mixture. It contains 23% of dicarboxylic acids and is digested by trypsin.

The amino acid composition of the three proteins of myelin is shown in Table I.

AUTOIMMUNITY

The autoimmune state arises when humoral antibody or sensitized cells are produced in a host and are directed against components of the host's tissues. When sensitized cells are involved, tissue damage usually occurs. These are a group of diseases involving the thyroid, testes, kidneys and joints and are known collectively as autoimmune disease. When only humoral antibody is involved, clinical symptoms rarely appear, unless the antibody is directed against unorganized tissue, such as the red blood cells. The antigens which cause autoimmunity may be present only in cells of a single organ, the so-called organ-specific antigens, or they may be present in all the organs of the body, in which case they are known as species-specific antigens.

Several theories have been postulated concerning the development of autoimmunity. According to one theory, autoimmunity is due to the breakdown of immunological tolerance to autoantigens. The loss of tolerance might come about by exposing the host to bacterial organisms or other exogenous materials that share antigenic determinants with tissue antigens. One example is the cross-reaction known to exist between Type 14 pneumococcus polysaccharide and human erythrocytes (61,62). The cross-reactivity between Type 12 hemolytic streptococci and rat kidney reported by Markowitz et al. (63) is another example. Another theory is the "sequestered" antigens concept.

TABLE I

AMINO ACID COMPOSITION (mole %) OF THE THREE MYELIN PROTEINS

Amino Acid	Wolfgram Proteolipid	Basic Protein	Folch-Pi Proteolipid
Lysine	6.95	7.79	4.3
Histidine	2.29	5.04	1.9
Arginine	5.83	9.47	2.6
Aspartic Acid	9.90	6.71	4.2
Threonine	5.16	3.71	8.5
Serine	5.84	9.46	5.4
Glutamic Acid	12.95	7.63	6.0
Proline	4.64	7.24	2.9
Glycine	8.00	15.49	10.3
Alanine	8.45	8.90	12.5
Half Cystine	1.01	0.00	4.2
Valine	5.84	1.38	6.9
Methionine	2.13	1.28	1.7
Isoleucine	4.27	1.57	4.9
Leucine	9.63	6.02	11.1
Tyrosine	2.87	2.79	4.7
Phenylalanine	4.22	5.36	7.8

Antigens, like the lens proteins, thyroglobulin, the proteins of spermatozoa, are produced after birth but remain sequestered from the circulation and do not normally come into contact with immunocompetent cells. If, as a consequence of trauma, burns or infection, such antigens get into the blood, they can stimulate immunocytes to engender specific antibodies against them (64). The third theory postulates that autoimmunity results from some abnormality in the functioning of the immune system (65).

As early as 1900, Metchnikoff (66) reported the induction in guinea pigs of antibodies directed against their own spermatozoa. After the development of Freund's adjuvant in 1943 (67), it was found that the immune response to normal tissue constituents could be induced with relative ease. Thus, autoantibodies directed against brain components could be produced provided that the brain extract was emulsified with Freund's adjuvant (68). Thyroglobulin also had to be emulsified in complete Freund's adjuvant in order to stimulate humoral antibodies in the homologous species (69). The mechanism by which Freund's adjuvant induces heightened immune response is not completely understood. It has been suggested that it might induce partial denaturation of the autoantigens and thus promote the immunogenicity of the antigen for the homologous species (70).

It should be noted that the demonstration of humoral autoantibodies in the serum does not indicate that they are responsible for the pathological changes in an organ. In general, it has been the case of the organ-specific autoimmune diseases, that there is no close correlation between the titre of humoral autoantibodies and the severity of

the disease. Indeed, in some instances, the possibility exists that they may be beneficial.

EXPERIMENTAL AUTOIMMUNE DISEASES

Autoimmunity is of current interest in the fields of medicine and most biological sciences and there are several books published dealing solely with this subject (71,72,73). In order to understand the mechanism underlying the human autoimmune diseases, experimental animals were used as models for this study. Freund's adjuvant must be used in order to induce most of the experiment autoimmune diseases. There has been increasing attention paid to the abnormal cellular reactivity induced by Freund's adjuvant (74), and to the effects of the adjuvant on the thymus gland (75). It is thought that Freund's adjuvant, by inducing a cellular reaction within the thymus or a lymphoid tissue "opens the door" as it were, to antigens, thus heightening the probability of the occurrence of autoimmunization. A recent review by Paraf (76) showed that adjuvant acts both on the antigen and on the cells involved in immunological responses.

In order that an experimental disease may be classified as an "autoimmune" disease, three characteristics must be fulfilled (77):-

(1) The particular organ or tissue under consideration must possess organ-specific antigenic activity and must be capable of inducing, in an experimental animal, a demonstrable immune response directed against the tissue or organ in question. The immune response may be humoral, consisting of a circulating antibody, or cellular,

manifested as a delayed-type hypersensitivity.

(2) The characteristic tissue damage must be largely restricted to the tissue or organ under study.

(3) The tissue damage must be produced preferably with the animal's own tissue and with the tissue from other members of the same species.

Among the experimental autoimmune diseases, the one involving the central nervous system has been studied most thoroughly.

EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS (EAE)

EAE is an autoimmune disease of the central nervous system readily produced in animals by the injection of central nervous system tissue emulsified in complete Freund's adjuvant. The autoimmune disease has been studied intensively because some of the clinical and histological features of the disease are similar to the clinical symptoms and pathological lesions found in the human demyelinating disease, multiple sclerosis. Brain and spinal cord taken from animals with EAE exhibit histological changes which include infiltration of the affected tissue by lymphocytes and other mononuclear cells, and increased vascular permeability. The clinical signs consist of weight loss, fecal incontinence and limb paralysis.

In 1933, Rivers et al. (78) reported that repeated intramuscular injections of either an aqueous emulsion or an alcohol-ether emulsion of rabbit brain produced an acute disease of the central nervous system in two out of eight monkeys. Other investigators

(79,80) confirmed these studies and showed that the lesions were characterized by destruction of myelin. The phenomenon was neglected until 1946, when Morgan (81) Kabat et al. (82) and Freund et al. (83) showed that the condition could be induced by a small number of injections of nervous tissue incorporated in Freund's adjuvant. Morgan (27) produced the disease in monkeys with three injections of monkey neural tissue and called the condition "allergic encephalomyelitis". The term "experimental allergic encephalomyelitis" or "EAE" has been adopted to describe this disease.

THE IMMUNOLOGICAL BASIS OF EAE

The following evidence indicates that EAE is an immunological disease:-

a) There is an interval between the injection of nervous tissue and the appearance of clinical symptoms. The latent period is required for the development of the immune reaction against nervous tissue antigens and for the subsequent tissue destruction within the central nervous system.

b) Freund's adjuvant, known to enhance the immune responses, exerts a potentiating effect on production of EAE.

c) Central nervous system tissues taken from animals with EAE exhibit the typical infiltration of lymphocytes and plasma cells that characterizes delayed hypersensitivity reactions.

d) Specific antibodies which fix complement with nervous tissue extracts may be

demonstrated in the sera of a high proportion of sensitized animals (84,85).

e) The disease can be transferred with living lymph node cells derived from donors sensitized to nervous tissue.

NATURE OF THE IMMUNE REACTION RESPONSIBLE FOR EAE

That EAE is a manifestation of delayed hypersensitivity and that the disease can be transferred with sensitized lymphoid cells was demonstrated by Paterson (86). Subsequent work by Stone (87) and Åstrom and Waksman (88) confirmed this observation. Waksman (89) showed that the earliest feature of the EAE lesion in the guinea pig and rabbit is the accumulation of lymphoid cells and histiocytes around small cerebral veins and in the adjacent nervous tissue, and that demyelination follows such cell infiltration. The same author had demonstrated earlier (90) that skin tests in rabbits which had received injections of rabbit nervous tissue and adjuvant, resulted in a local inflammatory reaction which reached a maximum in forty-eight to seventy-two hours, and was found to be grossly and histologically typical of delayed hypersensitivity. Thus, the immune response in EAE is mediated by the sensitized cells.

A strong objection to the view that circulating antibodies play a pathogenic role in EAE is the consistent failure to induce the disease passively by injection of serum obtained from animals with the disease (91). It has been argued that insufficient antibody was transferred or that additional factors were needed. Jankovic et al. (92) reported that intraventricular injection into normal guinea pigs of serum from animals

with EAE resulted in the development of clinical and pathological changes resembling those of mild EAE whereas injections of normal serum were without any such effect. In this respect, it is interesting to note that aspermatogenis, an autoimmune disease involving the testicular tissue, was also regarded as not being transferrable by serum. Recently, Wilson et al. (93) reported that it is possible to transfer aspermatogenesis with serum provided the recipient was first injected with Freund's adjuvant. Therefore, it might be possible to transfer EAE with serum from sensitized animals provided the correct route is used.

The exact immunological mechanism leading to EAE is still debatable. Recently, Spitler et al. (94) showed that there is dissociation between cellular immunity to the encephalitogenic basic protein and the production of EAE. They demonstrated that it is possible to produce EAE in animals without cellular immunity to the basic protein, and conversely, that it is possible to produce cellular immunity to basic protein in animals which do not develop the disease. They concluded that an appropriate combination of cellular immunity and antibody production is necessary for the production of EAE as pointed out by Paterson (77).

Alvord (95) has summarized the events leading to the development of EAE. The disease is caused by sensitized lymphoid cells which cross the central nervous system blood vessels to destroy the myelin sheaths. The lymphocytes and macrophages mature locally into immunoblasts and plasma cells which may secrete more antibodies at the local inflammatory site.

THE HUMORAL ANTIBODY RESPONSE IN EAE

Encephalitogenic basic protein is a weak antigen, in respect to its capacity to induce a humoral antibody response. There is clear indication that circulating antibodies are not involved in the pathogenisis of EAE. Two types of circulating antibodies have been described in animals following sensitization with nervous tissue and adjuvant. Bornstein and his associates (96,97,98) showed that sera from animals affected with EAE and those from patients with multiple sclerosis bring about demyelination in myelinated cultures of mammalian central nervous system. Other workers (99, 100) confirmed this observation. The antibody is directed against myelin and glial cells and is a 7S immunoglobulin and is complement dependent. The antibody has been found to bind to myelin sheaths and the surface of neuroglial cells by the indirect immunofluorescent antibody technique (101).

The other type of antibody, known as complement fixing anti-brain antibody was studied by Paterson and his associates (102). It is directed against a cerebroside, a lipid haptenic component of myelin. This antibody occurs in response to nervous tissue sensitization. It is a 19S immunoglobulin and loses its activity after storage at 4°C for 2 weeks.

Early observations by Thomas et al. (85) showed that EAE was less frequent and tended to be less severe in dogs whose sera contained high titres of anti-brain antibody. Disease appeared earlier and tended to be more severe in dogs without such antibody. Subsequent studies supported the view that the anti-brain antibody

response conceively might represent a protective function. Paterson (103) showed that serum containing high levels of anti-brain antibody had suppressive effects on EAE when passively administered to other sensitized rats. The antibody has not been demonstrated in patients with multiple sclerosis (104).

SUPPRESSION OR PREVENTION OF EAE

EAE can be inhibited in animals by several methods. Alvord et al. (105) have defined the following terms: -

1) Prevention means that the inhibiting treatment precedes the injection of the EAE inducing challenge.

2) Suppression means the inhibiting treatment is administered after the EAE inducing injection.

Ferraro and Cazzullo (106) observed that EAE could be prevented in guinea pigs by the injection of large amounts of nervous tissue. In the studies of Condie and Good (107), adult rabbits were given repeated injections of nervous tissue homogenates via the subcutaneous or intraperitoneal routes prior to intracutaneous challenge with spinal cord and adjuvant. In such animals, EAE was dramatically inhibited. They concluded that prevention of disease was due to inhibition of antibody formation due to antigen overloading.

Kies and Alvord (108) found that the killed tubercle bacilli in the complete adjuvant could also prevent EAE. The exact mechanism for inhibition is not clearly

understood. Alvord (105) and Einstein et al. (109) showed that EAE can also be inhibited by the injection of nervous tissue or the purified encephalitogenic basic protein emulsified in incomplete Freund's adjuvant before challenge. Circulating antibody is normally found in these treated animals.

Basic protein rendered non-encephalitogenic through chemical modification of the single tryptophan residue has been reported to inhibit EAE by Chao and Einstein (58), Eylar et al. (59) and Swanborg (110). Swanborg showed that pretreatment of animals with modified non-encephalitogenic basic protein emulsified with incomplete Freund's adjuvant completely prevented EAE induction. If the protective dose was given after sensitization, the incidence of clinical signs was low, but many of the guinea pigs had histologic signs of EAE. The nature of the protective mechanism mediated by antigen-induced inhibition of EAE is still not clear. The possibility that blocking antibody played a protective role has been dismissed by Lisak et al. (111). They reported that combined treatment with basic protein and methotrexate before challenge prevented the formation of antibody to basic protein but does not interfere with disease inhibition by basic protein.

Non-encephalitogenic basic proteins isolated from immature human brain and bovine spinal cord have been reported by Einstein et al. (109) to inhibit EAE induction in guinea pigs. Recently, the same author (112) showed that chemically modified encephalitogenic basic protein was also effective in suppressing EAE. The guinea pigs treated with modified encephalitogen after sensitization did not show clinical neurological

signs although some had mild histological lesions in their brains, a finding which has been reported by others (105,111). The author concluded that the encephalitogen has two important sites; the one containing tryptophan is required for disease induction and the other, lacking tryptophan, is required for protection or prevention of disease. This non-encephalitogenic modified basic protein could be of potential therapeutic value in the treatment of human demyelinating diseases if such diseases could be shown to be due to the development of hypersensitivity to nervous tissue. However, it should be borne in mind that although this peptide is non-pathogenetic in guinea pigs, it has been reported to be pathogenetic in rabbits (113). It is not known how the human organism would respond to the tryptophan-modified protein, i.e., whether it would be unresponsive, like the guinea pigs or responsive, like the rabbit.

Other means of inhibiting EAE include the use of immunosuppressive agents such as cyclophosphamide (114), X-radiation (115), anti-lymphocytic serum (116) and the anti-leukemic agent, L-asparaginase used by Khan et al. (117).

THE USE OF IMMUNOCHEMICAL METHODS IN STUDIES ON PROTEINS

The main value of immunochemical methods is that they are precise, sensitive and specific. Antibodies will react only with the antigen used for immunization or with molecules possessing groups which are sterically closely related to the determinant groups of the original antigen. The sensitivity of various immunological methods is in general very high. The interaction between antibodies and their homologous antigens

may be manifested differently in vivo and in vitro. The precipitin reaction is the most common in vitro demonstration of antibody-antigen interaction. The specific precipitate is formed when the large antigen and antibody molecules unite in multiple proportions and the aggregated complexes separate from solution as an insoluble, antigen-antibody precipitate. Increased knowledge of the physio-chemical principles governing the precipitin reaction led to the development of several quantitative methods involving specific precipitation in gels (118,119).

The double diffusion method of Ouchterlony (119) using agar in Petri dishes permits not only the determination of the number of precipitating systems but also the detection of identical antigens present in different solutions. Figure 1 shows three possible patterns:- (1) the precipitin bands opposite adjacent antigen wells fuse when the antigens in the wells are identical; (2) spur formation occurs at the junction of the bands opposite the antigen wells when the antigens are similar but not identical; (3) crossed precipitin bands when the wells contain different antigens.

One of the most useful technical developments in the field of Immunology is the method of immunoelectrophoresis, which was first described in 1953 by Grabar and Williams (120). In this procedure, electrophoretic separation in gels is followed by an Ouchterlony type of immunodiffusion reaction. The proteins in a mixture are first segregated according to their surface charges; later, they are identified and enumerated by allowing the specific antiserum to diffuse into the agar perpendicular to the direction of electrophoresis. The procedure is schematically illustrated in

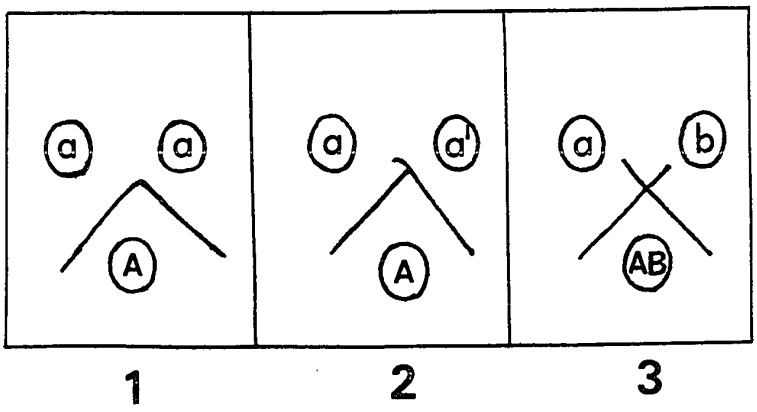


Figure 1. Ouchterlony plates. A and B are two antibodies directed against the antigens, a and b, respectively. a' is an antigen related to and cross-reacting with a.

1. reaction of identity
2. reaction of partial identity
3. reaction of unrelated antigen-antibody system

Figure 2. The main advantages of immunoelectrophoretic analysis are; (1) simplicity; (2) the possibility of analyzing complex antigenic mixtures; (3) each component can be defined or identified by its electrophoretic mobility.

Rao et al. (121) were among the first investigators to apply immunodiffusion method to the study of heterologous lens antibodies. MacPherson and Liakopoulou (4) have demonstrated the presence of a minimum of 12 antigens in saline extracts of homogenized rat brain by immunodiffusion and immunoelectrophoresis. With these methods, they were able to show that one of the species-restricted antigens of the rat brain, a SRANT protein, is found in fetal brain and disappears from the brain a few days after birth, while the concentrations of the other two SRANT proteins rise slowly after birth. By means of immunoelectrophoresis, normal human serum was shown 20 years ago to contain at least 15 different antigenic substances (122). Immunochemical methods have also been invaluable in the study of the surface antigens of blood and tissue cells; for monitoring purification of proteins; for determining the protein components of biological fluids; and for measuring the concentrations of biologically active substances such as hormones.

GROSS ANATOMY OF THE SPINAL CORD

The central nervous system consists of the brain and the spinal cord, which is a cylindrical mass of nervous tissue occupying the vertebral canal. It extends from the foramen magnum, where it is continuous with the medulla oblongata, to the level of

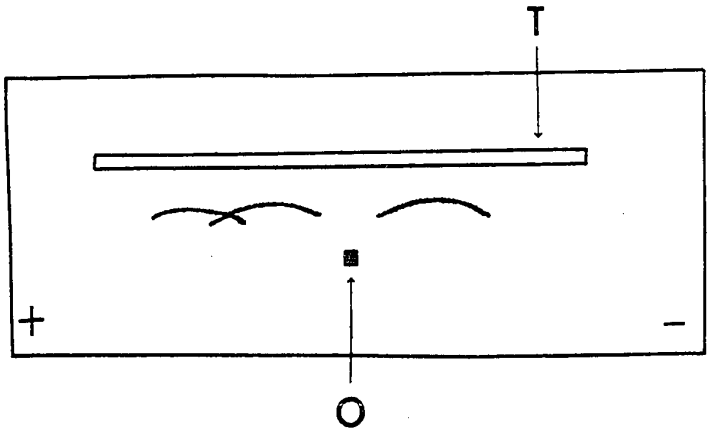


Figure 2. Schematic representation of immunoelectrophoretic analysis.

The protein mixture is applied at the origin, O. After completion of the electrophoresis, the trough T, in the gel is filled with an immune serum containing antibodies directed against the components in the protein mixture. The antibody and antigen diffuse toward each other and form a precipitin arc, the location of which is determined by the surface charge of the antigen.

the first or second lumbar vertebra. The spinal cord is protected by three membranes or meninges and surrounded by the cerebrospinal fluid (CSF). The dura mater, the most superficial of the three membranes covering the spinal cord, is a thick tough layer of dense collagenous tissue. The fragile arachnoid membrane lies beneath the dura mater, while the inner-most membrane, the pia mater, is applied directly to the surface of the cord. Between the arachnoid membrane and the pia is the subarachnoid space, containing the CSF (123, 124).

CROSS-SECTION OF THE SPINAL CORD

The body of the spinal cord is composed of gray and white matter. The gray matter forms a continuous fluted column in the centre of the cord and this is completely surrounded by the white matter. The gray matter contains nerve cell bodies and in cross-section has the form of a letter H (Figure 3). The white matter consists of myelinated nerve fibers, supported by the oligodendrocytes and astrocytes.

THE SCOPE OF THE PRESENT STUDY

There is very little information concerning the nature and number of proteins present in a spinal cord extract. The best known immunogen of spinal cord is the myelin basic protein that induces EAE. EAE has been used as an experimental model to study the human demyelinating disease, multiple sclerosis. EAE is the most widely studied autoimmune disease and has been shown to be due to hypersensitivity to basic

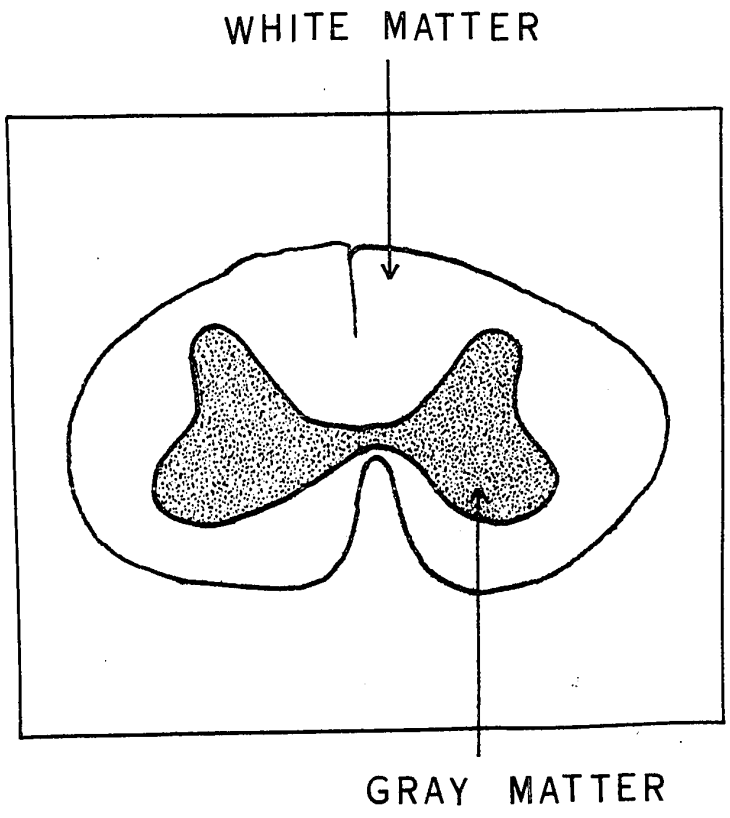


Figure 3. A cross-section of the spinal cord showing the gray and the white matter.

protein. It is still not known if multiple sclerosis is an autoimmune disease caused by hypersensitivity to human encephalitogenic basic protein.

The present investigation has been directed towards the isolation and partial characterization of a protein in the spinal cord which appeared to have the capacity to prevent the development of EAE. We reported earlier (125, 126), that several rabbits which remained healthy after receiving an EAE inducing injection of bovine spinal cord had high levels of humoral antibodies directed against a particular protein of the soluble fraction of bovine spinal cord. This protein has a regional distribution in the central nervous system as it is localized mainly in the spinal cord although it can be detected in highly concentrated extracts of cerebral white matter.

II. EXPERIMENTAL

MATERIALS

Bovine spinal cords, peripheral nerves and brains were obtained from the slaughter house within an hour after the animals had been killed. Adhering tissue was removed from the cords and they were stored at -20°C. Normal human spinal cord and brain tissue were provided by the Autopsy Service of the Royal Victoria Hospital, Montreal. Usually, the tissues were obtained within 12 hours of death from victims of accidents or heart attacks. Spinal cords, peripheral nerves and brains were removed from rats, mice and rabbits within a few minutes of death. When cerebral gray or white matter was desired, it was obtained by blunt dissection from fresh brain. All tissues were stored at -20°C.

Cytochrome c (horse heart), ribonuclease (bovine pancrease), and α-chymotrypsinogen A (bovine pancrease) were products of Sigma Chemical Co., St. Louis, Mo. Bovine serum albumin, (fraction V), and bovine γ-globulin (Fraction II), were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Sephadex G-100, Sephadex G-25, DEAE-Sephadex A-50, CM-Sephadex C-25, and Blue Dextran 2000, a high molecular weight dextran having an average weight of 2×10^6 and covalently bound to a blue dye, were manufactured by A.B. Pharmacia, Uppsala, Sweden. Carboxylmethyl cellulose (CM-52) was purchased from Whatman (W. and R. Balstron Ltd., England).

Cyanogum - 41 was a product of American Cyanamide Co., Wayne, N.J., U.S.A. It is a mixture of two monomers; acrylamide and N, N-methylenebis acrylamide.

N,N,N,N-tetramethylethylene diamine was purchased from Distillation Products Industries, Division of Eastman Kodak Company. Dialyzing tubing No. 20 was manufactured by Union Carbide Company of Canada, Ontario. All other chemicals were reagent grade.

The collodion membranes used for concentration by positive pressure dialysis were obtained from Membranfiltergesellschaft, Göttingen, Germany. Freund's adjuvant (incomplete and complete) and Mycobacterium butyricum were obtained from Difco. New Zealand white rabbits (female), male outbred guinea pigs and Wistar rats were purchased from Canadian Breeding Farm and Laboratories Ltd., Quebec, Canada.

METHODS

1. PREPARATION OF ORGAN EXTRACTS

a) Bovine Spinal Cord Extracts

Bovine spinal cords (BSC) from which the meninges had been stripped were minced and added to a sufficient volume of chilled 0.1 M NaCl to make a 10% suspension. The suspension was homogenized at 0°C. in a Tri-R homogenizer (Tri-R Instruments, Jamaica, New York), at 4,500 rpm for 2 minutes. The homogenates were then centrifuged in an International Model B-20 centrifuge at 40,000 x g at 0°C for 40 minutes. The supernatants were removed and the precipitates were resuspended in half the original volume of cold 0.1 M NaCl and centrifuged again. The precipitate was discarded and the supernatants, called BSC extracts, were concentrated to 20 mg protein per ml at

4°C in dialysis tubing under a positive pressure of 15 cm of Hg by the method of Mies (127). It was stored at -20°C.

b. Extracts Of Mammalian Spinal Cord Of Other Species

When extracts of other mammalian spinal cords were needed, the frozen tissues were processed as described above for bovine spinal cords.

c. Dialysis Tubing Filtrate (DTF)

The filtrate obtained from the concentration of BSC extract in dialysis tubing was collected and concentrated by flash evaporation to contain about 15 mg protein per ml.

2. PREPARATION OF SUBCELLULAR FRACTIONS FROM BOVINE SPINAL CORD

Preparation of the crude nuclear, mitochondrial, microsomal and soluble fractions of bovine spinal cord was carried out by differential centrifugation according to the method of De Robertis et al. (128). Briefly, the minced bovine spinal cord tissue was added to sufficient 0.32 M sucrose containing 0.02 M Tris-HCl buffer, pH 7.6 and 3×10^{-3} $MgCl_2$ to make a 10% (W/V) suspension and homogenized at 0°C in a Tri-R homogenizer at 4,500 rev/min for 2 minutes using a glass-teflon pestle. The homogenate was centrifuged in successive stages at 900 x g for 10 minutes, 11,500 x g for 20 minutes and 100,000 x g for 30 minutes. The precipitates corresponded to the crude nuclear (N), mitochondrial (MIT) and microsomal (MIC) fractions. The pellets were washed twice

by resuspending them in five times their volume of the 0.32 M sucrose solution and re-centrifuging them at the speed at which they had been collected. The washed pellets were suspended in 0.1 M NaCl to make a 20% suspension, stirred at 4°C for one hour and then centrifuged in an International Model B-20 centrifuge at 40,000 x g at 0°C for 40 minutes. The precipitates were washed once with 0.1 M NaCl and recentrifuged. The supernatants from each pellet were combined, concentrated by ultrafiltration to 20 mg protein per ml and tested for spinal cord protein by immunodiffusion analyses. The pellets were then defatted by mixing them five times with 8 volumes of acetone at -10°C. The final residue was dried by gentle mixing with a spatula on blotting paper under the fume hood. The defatted powders were then extracted first with 0.1 M NaCl and then with 0.1 M Na-citrate buffer, pH 4.3. All extracts of the washed particulate fractions were concentrated by ultrafiltration in dialysis tubing to about 6-10 mg protein per ml. They were tested by immunodiffusion analysis for the presence of spinal cord protein.

3. PREPARATION OF ANTISERA

The anti-bovine SCP sera used during the initial stages of this research were obtained from rabbits that had been immunized with 250 mg of BSC emulsified in 0.5 ml of complete Freund's adjuvant. Later, rabbits were immunized with 6 mg of partially purified saline extracts of BSC or 3 mg of purified β_1 or γ-SCP emulsified in complete Freund's adjuvant. The emulsion was injected into the hind foot pads and the animals

were bled four weeks later from the marginal ear veins. Rabbits that had produced an acceptable level of humoral anti-SCP antibodies were reinjected as above and bled at monthly intervals. The antisera were stored at -20°C.

4. ABSORPTION OF ANTISERA

The elimination of undesirable antibodies from the antisera was accomplished by the technique of absorption. Antibodies to bovine serum (BS) proteins were removed from antisera by adding 0.05 ml of BS to each 1.0 ml of antiserum. The mixture was allowed to stand at 37°C for half hour. After 30 minutes, the specific precipitate was removed by centrifugation. Absorptions were repeated until immunodiffusion analyses indicated that all the antibodies to serum proteins had been removed. Usually two absorptions with bovine serum were sufficient to remove all the antibodies directed against the serum proteins. Antibodies to bovine nervous tissue antigens other than SCP were removed by absorbing the antisera as above with extracts of bovine brain gray matter or selected fractions obtained in the course of purification of SCP by column chromatography.

5. BATCH ABSORPTION OF BSC EXTRACTS ON DEAE-SEPHADEX A-50

Ten g of DEAE-Sephadex A-50, previously equilibrated with 0.05 M Tris-HCl buffer, pH 7.0, was stirred into a sample of BSC extract that contained 1.0 g of protein and had been desalted on a Sephadex G-25 column with the same buffer. After 2 hours, the DEAE-Sephadex A-50 was separated by centrifugation and the supernatant was

concentrated to 25 mg protein per ml by flash evaporation.

6. CHROMATOGRAPHY OF PARTIALLY PURIFIED BSC EXTRACT ON CM-52 CELLULOSE

The BSC extract which had been absorbed with DEAE-Sephadex A-50 was desalted on a Sephadex G-25 column with 0.05 M sodium acetate buffer, pH 5.5 and then applied to a CM-52 cellulose column (1.7 cm x 70 cm) which had been equilibrated with the same buffer. The proteins were eluted with a stepwise gradient of NaCl in the pH 5.5 buffer. The protein concentration of the eluates was determined at 280 nm in a Hitachi UV-VIS Spectrophotometer.

7. MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION

Sephadex G-100 was equilibrated and allowed to swell in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M KCl for 48 hours. A column (1.5 x 80 cm) was packed and calibrated in the manner described by Andrews (129) using proteins of known molecular weight as standards. The proteins were dissolved separately in the eluting buffer at a concentration of 2 mg per ml and applied under the eluant to the surface of the gel bed. The proteins were eluted at the rate of 6.0 ml per hour, and collected in 1.0 ml fractions. The concentration of cytochrome c was determined at 412 nm. Elution volumes, Ve, corresponding to the maximum concentrations of the proteins, were estimated to the nearest 1.0 ml from the elution diagram. The void volume of the column was determined

using Blue Dextran 2000.

8. POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

Polyacrylamide disc gel electrophoresis was performed according to the method described by Davis (130), except that the sample and spacer gels were omitted. The sample, containing from 50 to 200 µg of protein in approximately 0.1-0.2 ml was applied directly on top of the running gel and the electrode buffer was carefully layered over the sample. A constant current of 2 ma per tube was applied until the dye had entered the gel; then the current was increased to 2.5 ma per tube until the bromphenol blue dye had travelled 6 cm in the gel. Gels were removed from their tubes, stained with 1% amido black -10B in 7% acetic acid for one hour. The gels were destained electrophoretically in 7% acetic acid.

9. DETERMINATION OF MOLECULAR WEIGHT BY DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

The molecular weight of the purified SCP was also determined by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate as described by Weber and Osborne (131). Proteins of known molecular weight were used as standards. The samples were incubated at 37°C for 2 hours in a solution containing 0.01 M sodium phosphate buffer, pH 7.0, 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. For electrophoresis, samples containing from 10 to 50 µg of protein in 0.05 to 0.1 ml were mixed with 3 µl of 0.05% bromphenol blue, 5 µl of 2-mercaptoethanol, 40 µl of glycerol, and 50 µl of

dialysis buffer (131). The mixtures were applied directly to the top of the 10% gel and the electrode buffer was carefully layered on top of each sample to fill the tubes. Electrophoresis was performed at a constant current of 8 ma per gel with the positive electrode in the lower electrode chamber. Under these conditions the marker dye moved 6 cm through the gel in approximately 5 hours. After electrophoresis, the gels were removed from the tubes and the length of the gel and the distance travelled by the tracking dye were measured. The gels were stained with 1% amido black in 7% acetic acid for 2 hours. Unbound stain was removed electrophoretically in 7% acetic acid. Gels swelled some 5% in the acidic solution used for staining and destaining (131). Therefore, the calculation of the mobility has to include the length of the gel before and after staining as well as the mobility of the protein and of the marker dye. The mobility of the protein was calculated using the following formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

The mobilities were plotted against the known molecular weights expressed on a semi-logarithmic scale.

10. AMINO ACID ANALYSIS

The protein sample was hydrolyzed with twice glass-distilled constant boiling (5.7 N) hydrochloric acid in an evacuted sealed pyrex tube according to the method of Stein and Moore (132). The hydrolysis was conducted at 110°C for 24 hours. The

hydrolysate was evaporated under vacuum with a bath temperature of 40°C and the excess HCl was removed by repeated evaporation after the addition of water. The final residue was quantitatively transferred to the amino acid analyzer column. The amino acid analysis was performed in a Beckman 120 B automatic amino acid analyzer (I would like to thank Dr. L. Goodfriend, Division of Immunochemistry and Allergy, Royal Victoria Hospital, Montreal, for performing this analysis.)

11. ASSAY OF ANTI-SCP ANTIBODIES BY THE PASSIVE HEMAGGLUTINATION (PHA) TECHNIQUE

The titres of anti-SCP antibodies in the rabbit and guinea pig sera were determined by a modified version of the micro PHA test using bis-diazotized benzidine (BDB). BDB used for coupling the antigen to the sheep erythrocytes was prepared according to the method of Frick (133). Briefly, BDB was prepared by dissolving 0.3214 g of benzidine dihydrochloride in 45 ml of 0.1 N HCl and the mixture was cooled to 0°C. A solution of $NaNO_2$ (prepared by dissolving 0.175 g $NaNO_2$ in 5 ml of distilled water) was added slowly to the mixture and the reaction was allowed to proceed for 30 minutes with intermittent stirring. The solution was then distributed to 2 ml vials, which were quick frozen at -78°C, sealed and stored at -20°C until used. When required, the solid BDB solution was melted by rotating the vials in the palm of the hand; it was then added to 14 ml of cold phosphate buffer, pH 7.3, and used for coupling the antigen to the sheep erythrocytes.

a) Standardization Of The Method

To establish the optimal ratio of BDB to antigens for each batch of BDB, the following procedure was adopted. Maintaining the volume of erythrocytes constant, (i) the BDB-phosphate volume was varied, keeping the antigen concentration constant, (ii) the antigen concentration was varied and the BDB-phosphate volume was kept constant. The proportions of BDB-phosphate solution to antigen were considered optimal when the highest titres were obtained with immune sera and when no reaction ensued with the normal sera.

b) Sensitization Of The Erythrocytes

Sheep red blood cells were washed 3 times by resuspending them in cold saline, and the washed packed cells were finally resuspended in an equal volume of physiological saline. The optimal quantity of antigen was placed in a 15 ml centrifuge tube and mixed with 0.1 ml of a 50% suspension of red cells. The optimal amount of BDB-phosphate solution was then added to the mixture, and the reaction was allowed to proceed at room temperature for 15 minutes with occasional gentle shaking. "Sensitized" cells prepared in this way were separated by centrifugation and the brownish supernatant was discarded. The cells were washed with 3.5 ml of diluent and recentrifuged. Finally, sensitized red blood cells were resuspended in 5 ml of diluent to make a 1% (v/v) suspension.

c) BDB Haemagglutination Test

The micro-haemagglutination test was performed with the Microtiter Kit manufactured by Cooke Engineering Company, Alexandria, Virginia, U.S.A. The method was essentially the same as Takasy's (134). Plexiglass plates with v-shaped bottom, having 96 wells arranged in 8 rows of 12 wells were used. To remove non-specific agglutinins, all antisera were absorbed for one hour at room temperature with an equal volume of packed washed erythrocytes. A calibrated dropper was used to place 0.025 ml of diluent into each well. Immune sera were added to the first row of wells and were serially diluted two-fold using stainless steel loops calibrated to deliver 0.025 ml. Then one drop (0.025 ml) of the suspension of sensitized cells was added to each well. The plates were gently shaken to ensure even distribution of the cells and the reaction was allowed to proceed at room temperature. The titre of the antiserum was expressed as the reciprocal of the highest dilution of antiserum which gave a positive pattern.

12. IMMUNOCHEMICAL ANALYSES

a) Double Diffusion Analyses

Ouchterlony (119) type analyses were carried out on glass microscope slides. The slides were covered with agar (1.3%) dissolved in 0.075 M barbiturate buffer, pH 8.3. The wells were cut in the solidified gel about 0.5 cm apart in a suitable pattern. The antigen solution and the antiserum were then placed in the appropriate wells. The

plates were left at room temperature for 12 to 24 hours, and the results were recorded. Then the slides were washed for 24 hours in 3 changes of saline at 4°C, photographed, dried and stained with acid fuchsin.

b) Immunoelectrophoresis

Immunoelectrophoresis (135) was carried out according to the method of Wieme and Rabaeye (136), using glass microscope slides prepared in the same manner as for double diffusion analyses. Electrophoresis was carried out at 15 ma per slide for 40 minutes at room temperature using 0.075 M barbiturate buffer, pH 8.3, in the electrode compartments. When electrophoresis was terminated, antiserum was added to a trough cut parallel to the path of electrophoretic migration. The slides were left at room temperature for 12 to 24 hours, before the patterns were drawn. The slides were then washed for 24 hours in 3 changes of saline at 4°C, photographed, dried and stained with acid fuchsin.

c) Localization Of Antigenic Proteins After Electrophoresis In Polyacrylamide Gel

After completion of the electrophoresis in polyacrylamide gel, the position of an antigenic protein was located by placing the gel in a slit cut out from a slab of buffered 1.3% agarose. Melted agarose was then carefully poured around the gel so as to remove all air bubbles. A second trough was cut 5 mm away from the polyacrylamide gel strip and this trough was filled with antiserum. The plates were left at room temperature for 24 hours to 48 hours until the precipitin lines developed. The mid-point of the

precipitin arc was taken to indicate the position of the band of the homologous protein in the gel.

13. PROTEIN DETERMINATION

The protein concentration was determined by the method of Lowry et al. (137) using bovine γ-globulin (fraction II) as the standard.

14. HEAT INACTIVATION

Heat inactivation of the antigens in BSC extract was accomplished by immersing a tube containing the BSC extract in a boiling water bath for 30 minutes. The precipitate was removed by centrifugation and the antigens left in the supernatant were detected by the double diffusion analyses.

15. QUANTITATION OF SCP BY SINGLE RADIAL IMMUNODIFFUSION

The quantitation of SCP by single radial immunodiffusion was carried out according to the method of Mancini et al. (138). One ml of the monospecific anti-SCP serum, heated in a water bath at 55°C, was added to 11 ml of 1.2% agar in barbiturate buffer, held at the same temperature. The mixture was pipetted onto a warm glass plate (10.2 x 8.6 cm). After the gel had solidified rows of circular wells spaced 1.5 cm apart were punched out, using a No. 12 hypodermic needle from which bevel had been cut off. Standard solutions of purified β_1-SCP were prepared which contained 0.2 mg,

0.15 mg, 0.1 mg, 0.075 mg and 0.05 mg protein per ml. When the appropriate wells had been filled with the standard solutions and the solutions to be tested, the agar plate was stored in a moist chamber at room temperature for 24 to 48 hours. To obtain the standard calibration line, the diameters of the circles formed around the wells containing $ß_1$-SCP were measured and plotted on the abscissa against the concentrations of the $ß_1$-SCP solutions on the ordinate. Concentrations of SCP in BSC extracts, in the DTF of BSC extracts were calculated from the standard calibration graph using the ring diameters formed by the respective solutions.

16. ASSAY OF ENCEPHALITOGENICITY OF $ß_1$-SCP

Random bred male guinea pigs weighing 300-500 g were used to assay the encephalitogenicity of $ß_1$-SCP. Guinea pigs were sensitized with 0.3 ml of an inoculum prepared by emulsifying 0.15 ml of saline containing 300 µg of $ß_1$-SCP and an equal volume of complete Freund's adjuvant containing 4.5 mg of Mycobacterium butyricum per ml. Each animal received 0.1 ml of emulsion into each hind foot pad and into the skin over the sternum. The animals were examined daily for clinical signs of EAE.

17. BIOLOGICAL ASSAY FOR THE ANTI-ENCEPHALITOGENIC ACTIVITY OF SCP

The anti-encephalitogenic activity of SCP was studied in both actively and passively immunized animals.

a) Induction Of EAE

Random bred male guinea pigs weighing 300-500 g were used. They were challenged with 0.3 ml of an inoculum prepared by emulsifying the appropriate amount (wet weight) of BSC or bovine basic protein (a gift of Dr. Marian W. Kies, Section on Myelin Chemistry, National Institute of Mental Health, Bethesda, Maryland, U.S.A.) in 0.15 ml of saline with 0.15 ml of complete Freund's adjuvant containing 4.5 mg of Mycobacterium butyricum per ml. Each animal received 0.1 ml of emulsion into each hind foot pad and into the skin over the sternum. Bovine spinal cord was titrated at levels of 15 mg, 30 mg and 100 mg and basic protein was titrated at 10 µg and 20 µg levels to determine their encephalitogenicity under our experimental conditions. The animals were weighed and examined daily for loss of weight, weakness, fecal incontinence, ataxia, impaired righting reflexes, and paralysis of the hind legs. Guinea pigs that developed paralysis of the hind legs were killed. In some animals, onset of death was acute and occurred overnight although there had been no warning signs of illness at the time of examination on the previous day. Apparently healthy animals were sacrificed from 28 to 30 days after challenge. The brains and spinal cords were fixed in 10% neutralized formalin. Tissue sections were cut from multiple levels from mid-brain and brain stem; cross-sectional and longitudinal slices were cut from the spinal cord. The sections were stained with hematoxylin and eosin and Luxol fast blue. Histological changes included cellular infiltration and perivascular cuffing. (I would like to thank Dr. W. Thelmo of the Department of Pathology, McGill University for performing the histology.)

b) Proteins Tested For Protective Activity

The proteins tested for protective activity included β_1-SCP and γ-SCP isolated from BSC, bovine brain gray matter extract and bovine liver extract. The preparation of BSC extract has been described. Bovine brain gray matter was obtained by blunt dissection from brains obtained within an hour of death by exanguination. A 10% homogenate of the minced gray matter in 0.1 M NaCl was centrifuged at 0°C for 40 minutes at 40,000 x g. The supernatant was concentrated by ultrafiltration to contain 15 mg of protein per ml. An extract of bovine liver was prepared in the same fashion.

c) Active Protection

Groups of 6 randomly bred male guinea pigs weighing 300-500 g were used. The total volume of each dose of inoculum was 0.1 ml and consisted of a saline solution containing the appropriate amounts of the protein to be tested emulsified with an equal volume of incomplete Freund's adjuvant. One-half of the inoculum was injected into each hind foot-pad and three injections were administered at weekly intervals. Thus, the total β_1-SCP injected per animal was 0.3 mg while a total of 15 mg of bovine brain gray matter protein or bovine liver protein was injected into each animal in the two control groups. Seven days after the last injection the animals were challenged with an EAE inducing inoculum of 100 mg (wet weight) of BSC or 100 µg bovine basic protein emulsified in complete Freund's adjuvant. The animals were observed daily for the onset of the disease as described above.

d) Preparation Of Anti-β_1-SCP Serum

A pool of anti-β_1-SCP serum was raised in guinea pigs by the injection of 0.1 mg of β_1-SCP in incomplete Freund's adjuvant. The inoculum was injected weekly into the hind foot pads. A total of 3 to 4 injections were given. Sera were obtained by cardiac puncture and were tested for anti-β_1-SCP antibody by immunodiffusion analyses. Those animals that had engendered a suitable level of antibody were reinjected and bled at monthly intervals. The antisera were pooled and the titre determined by the passive haemagglutination test.

e) Administration Of Anti-β_1-SCP Serum To Guinea Pigs

Preliminary experiments were carried out to determine the dilution, rate of absorption and elimination of guinea pig antibodies administered to guinea pigs. Male guinea pigs were injected intraperitoneally with 2 ml of an anti-β_1-SCP serum (PHA titre, 5120). Blood samples were withdrawn by cardiac puncture at 4 hours, 1, 3, 8, 12, 14, 23 and 33 days after the administration of the antiserum. The concentration of anti-β_1-SCP antibody in each sample was determined by the passive haemagglutination test.

f) Passive Protection

Random bred male guinea pigs weighing 400-500 g were used. Each received an intraperitoneal injection of 4 ml of a pooled guinea pig anti-β_1-SCP serum, PHA titre, 5120, four hours before challenge with 30 mg of BSC or 20 µg of bovine basic protein

emulsified in complete Freund's adjuvant containing 4.5 mg Mycobacterium butyricum per ml. The animals were reinjected with 2 ml of anti-β_1-SCP serum every 3 or 4 days in an endeavor to ensure that the anti-β_1-SCP PHA titre was maintained at about 250. Control animals received 4 cc of normal guinea pig serum intraperitoneally and were challenged as above. Animals were observed daily for the onset of disease and histological studies were made as described.

III. RESULTS

ISOLATION OF BOVINE SPINAL CORD (BSC) PROTEINS

In the early stages of this work, the spinal cord protein, abbreviated as SCP, was extracted with 0.1 M sodium citrate buffer, pH 4.3, from BSC that had been defatted with acetone according to the method of Kibler (139). Later, it occurred to us that the pretreatment of BSC with acetone might have denatured some of the proteins of the spinal cord. In the course of investigating this possibility, it was found that all of the SCP could be removed from undefatted cord by extraction with 0.1 M NaCl. It was observed that after BSC was extracted 18 times with 0.1 M NaCl, a total of 3.1 g protein was obtained from 100 g of BSC. This represents 3.1% of the original wet weight of the BSC. When the precipitate that remained after exhaustive extraction with 0.1 M NaCl was defatted with acetone and re-extracted with 0.1 M NaCl or 0.1 M sodium citrate buffer, pH 4.3, no significant amount of SCP was obtained.

PREPARATION OF ANTISERA

In the beginning of the present study, it was observed that several rabbits which had been injected with an EAE inducing dose of BSC in complete Freund's adjuvant did not develop encephalomyelitis. Moreover, their sera were found to contain antibodies directed against some antigenic components of the BSC. These antibodies were not detected in the sera of rabbits that had developed encephalomyelitis. The observation suggested that the antibodies elicited by spinal cord proteins could have been responsible for the suppression or prevention of EAE in the refractory animals. Later, when the

potential importance of SCP was apparent, rabbits were injected with BSC extract that had been absorbed with DEAE-Sephadex A-50. Antisera obtained from these rabbits contained antibodies directed against SCP as well as against other cationic antigenic proteins of BSC. In addition, the antisera also contained antibodies directed against the γ- and ß-globulins of bovine serum. These antisera were absorbed with normal bovine serum and are designated as "absorbed anti-D-BSCE sera". The antisera were used initially to monitor the purification of the predominant immunogen of BSC extracts. Later, when pure SCP was available, it was possible to prepare mono-specific anti-SCP rabbit serum which contained only antibody to the SCP.

ANTIGENIC PROTEINS OF BSC

When the BSC extracts were subjected to double diffusion analyses with absorbed anti-BSC serum, 5 to 7 precipitin bands could be counted (Figure 4). However, most of the antibody in the antiserum was directed against one antigen. This protein formed the heaviest precipitin band and was designated the spinal cord protein or (SCP). It will be seen from Figure 5, that the BSC extracts could be diluted to a concentration of 0.1 mg of protein per ml, and still contain sufficient SCP to form a line in immunodiffusion tests.

When BSC extracts were subjected to immunoelectrophoretic analysis at a concentration of 16 mg protein/ml (Figure 6) 6 to 7 precipitin arcs were observed opposite the upper well. Three to four proteins had the electrophoretic mobility of a

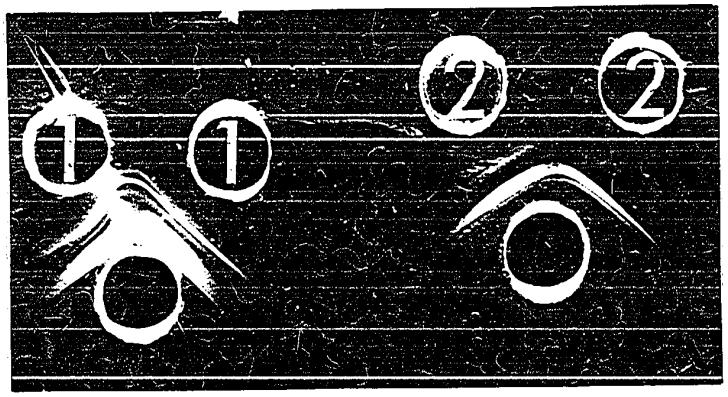


Figure 4. Immunodiffusion analyses of BSC extracts. Well (1) was filled with BSC extracts at a concentration of 10 mg of protein per ml. Well (2) was filled with BSC extracts at a concentration of 6 mg protein per ml. The lower well contained an absorbed rabbit anti-BSC serum.

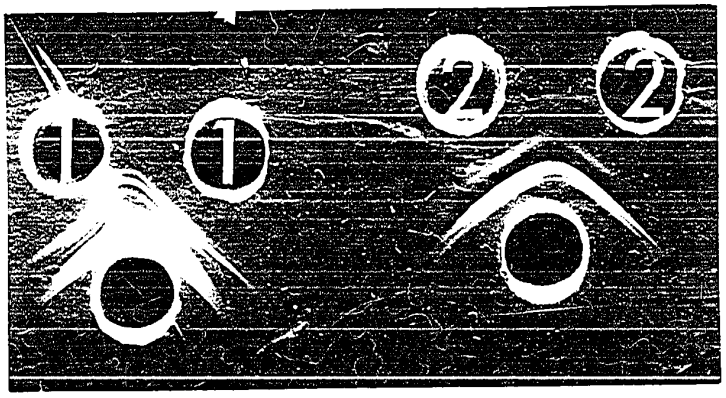


Figure 4. Immunodiffusion analyses of BSC extracts. Well (1) was filled with BSC extracts at a concentration of 10 mg of protein per ml. Well (2) was filled with BSC extracts at a concentration of 6 mg protein per ml. The lower well contained an absorbed rabbit anti-BSC serum.

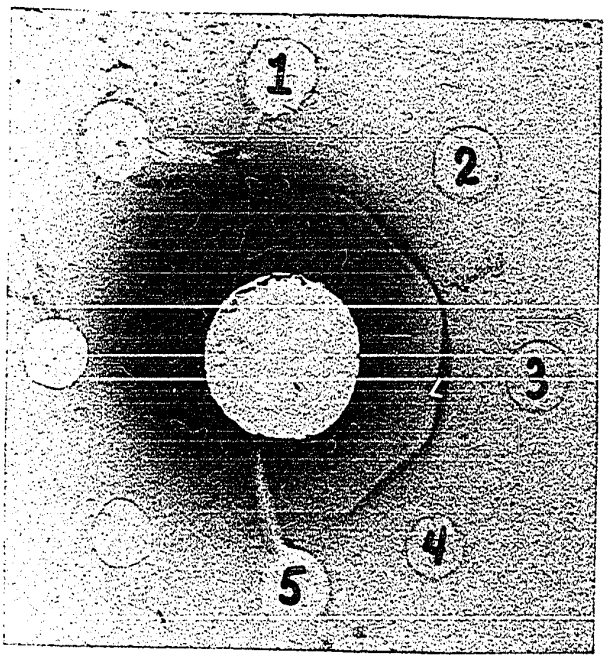


Figure 5. Immunodiffusion analysis of BSC extracts at various concentrations. Peripheral wells (1) to (5) were filled with BSC extracts containing 2.0, 1.0, 0.5, 0.25 and 0.12 mg of protein per ml, respectively. An absorbed rabbit anti-BSC serum was in the centre well.

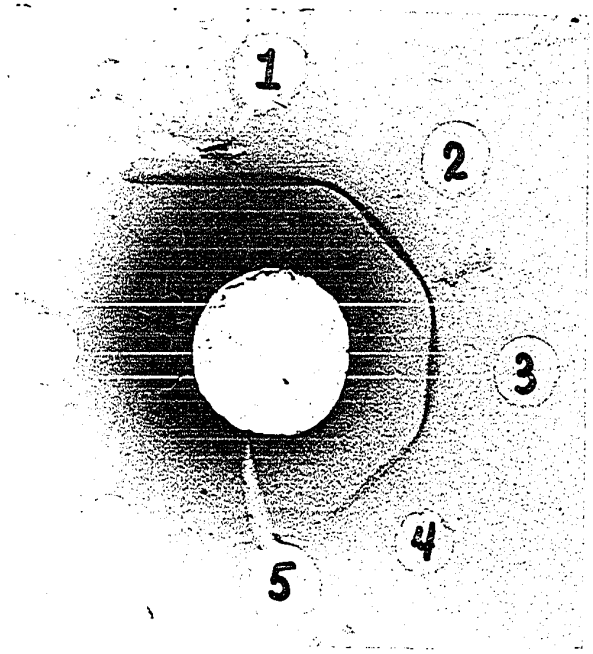


Figure 5. Immunodiffusion analysis of BSC extracts at various concentrations. Peripheral wells (1) to (5) were filled with BSC extracts containing 2.0, 1.0, 0.5, 0.25 and 0.12 mg of protein per ml, respectively. An absorbed rabbit anti-BSC serum was in the centre well.

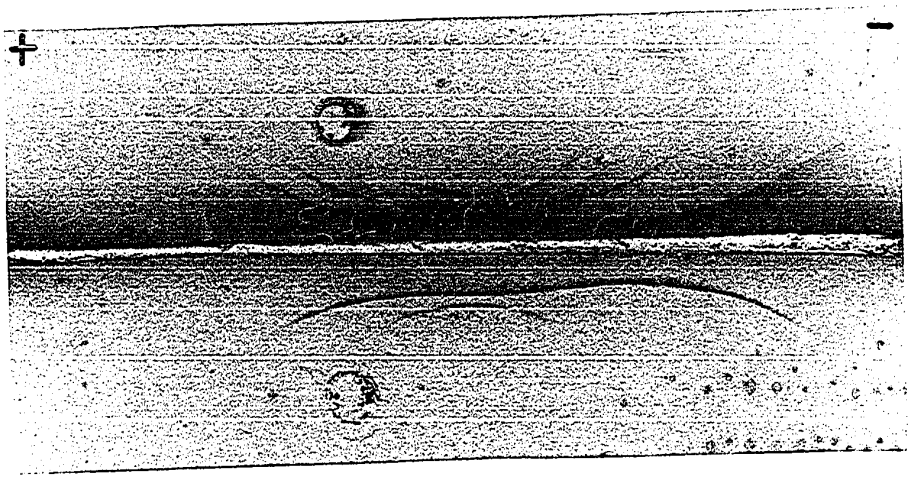


Figure 6. Immunoelectrophoretic analysis of BSC extracts. Upper well contained BSC extracts at a concentration of 16 mg protein per ml. Lower well contained BSC extracts at a concentration of 8 mg protein per ml. The centre trough was filled with an absorbed rabbit anti-BSC serum.

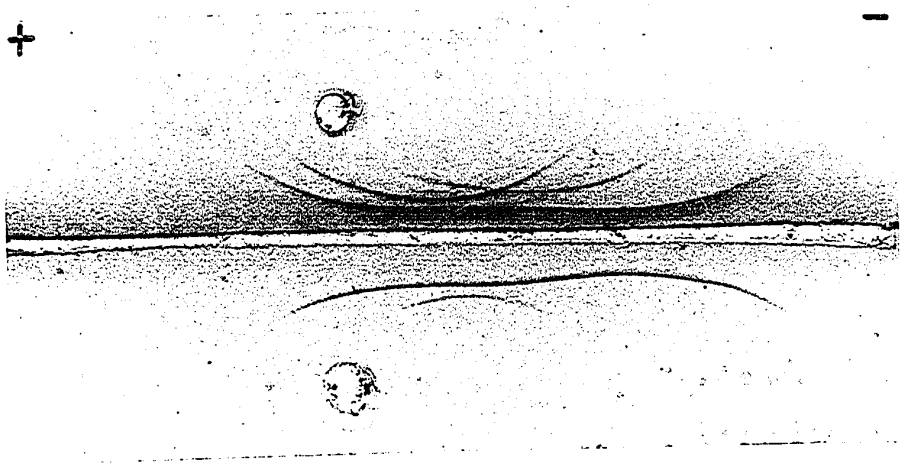


Figure 6. Immunoelectrophoretic analysis of BSC extracts. Upper well contained BSC extracts at a concentration of 16 mg protein per ml. Lower well contained BSC extracts at a concentration of 8 mg protein per ml. The centre trough was filled with an absorbed rabbit anti-BSC serum.

ß-globulin. One of these fused completely with a protein with a γ-globulin mobility. When the BSC extract was run at a concentration (lower well) of 8 mg protein per ml, the double arc was clearly visible while most of the other precipitin arcs were not visible. The double arc indicated that the SCP existed in two molecular forms having different electrophoretic mobilities.

ANTIGENIC PROTEINS IN THE DIALYSIS TUBING FILTRATE (DTF) OF BSC EXTRACTS

In general, proteins of spinal cord having molecular weights below 10,000 were not retained by the dialysis tubing and were collected in the filtrate. Several bands were observed (Figure 7) in the polyacrylamide gel electrophoretogram of the DTF obtained from the concentration of BSC extracts. When the DTF was subjected to double diffusion analysis, with absorbed anti-BSC serum, a single precipitin band was observed which fused completely with the line formed by the SCP retained by the dialysis tubing (Figure 8). This indicates that SCP also occurs in a third molecular size that is immunologically identical by immunodiffusion analysis to the major, large form of the molecule.

Immunoelectrophoretic analysis of the DTF revealed also that it contained only one antigen and that this antigenic protein had the electrophoretic mobility of a γ-globulin. The analysis is presented in Figure 9.

It has been noticed by other workers that brain proteinases were responsible for cleavage of proteins during homogenization of nervous tissue. Kies (140) suggested the use of chloroform-methanol (2:1) for homogenization because it was

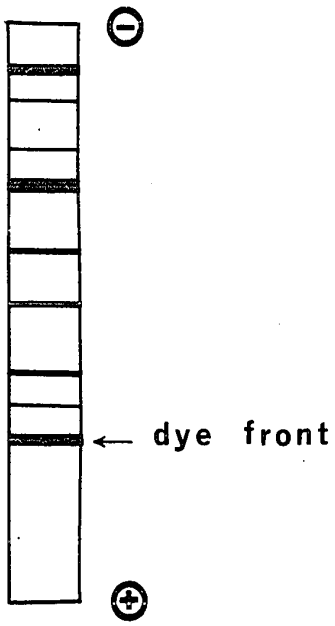


Figure 7. Polyacrylamide gel electrophoretogram of dialysis tubing filtrate (DTF) of BSC extracts.

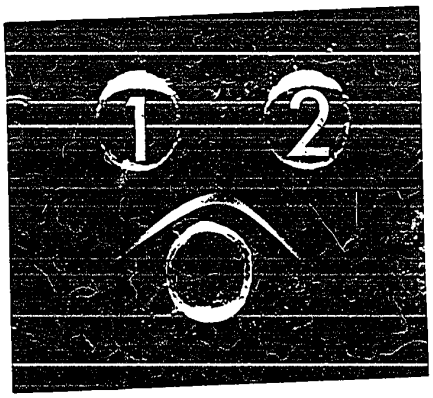


Figure 8. Immunodiffusion analyses of BSC extracts and DTF of BSC extracts.

Upper wells contained

(1) BSC extracts at a concentration of 1 mg protein per ml.

(2) DTF of BSC extracts at a concentration of 2 mg protein per ml.

The lower well was filled with an absorbed rabbit anti-BSC serum.

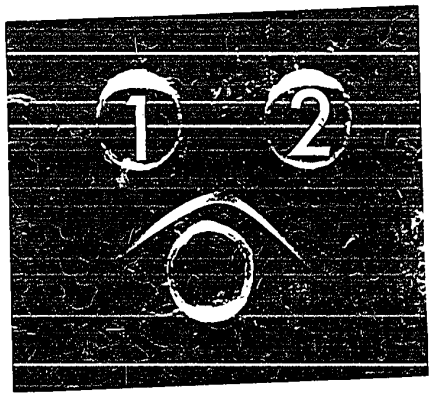


Figure 8. Immunodiffusion analyses of BSC extracts and DTF of BSC extracts.

Upper wells contained

(1) BSC extracts at a concentration of 1 mg protein per ml.

(2) DTF of BSC extracts at a concentration of 2 mg protein per ml.

The lower well was filled with an absorbed rabbit anti-BSC serum.

Figure 9. Immunoelectrophoretic analysis of BSC extracts and the DTF. The upper well contained BSC extracts at a concentration of 8 mg of protein per ml and the lower well contained DTF at a concentration of 13 mg of protein per ml. The centre trough was filled with an absorbed rabbit anti-BSC serum.

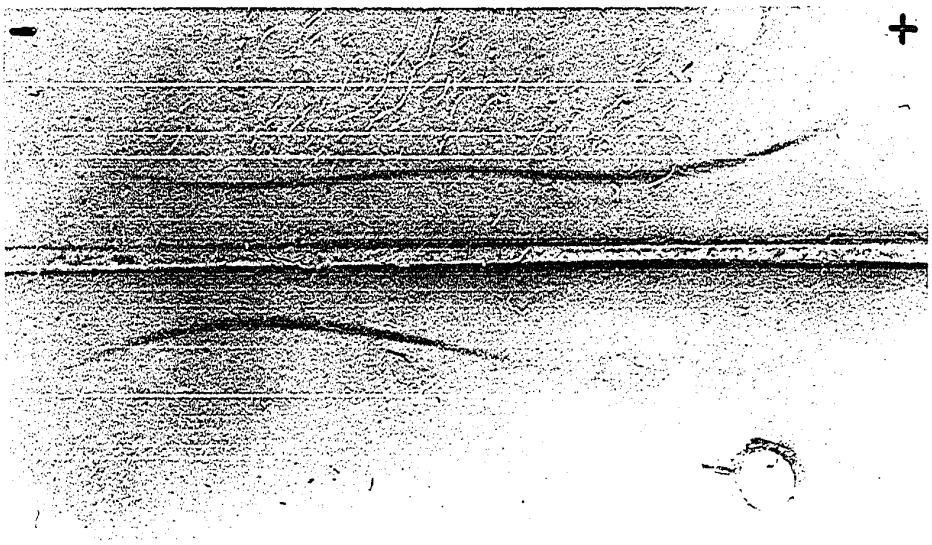


Figure 9. Immunoelectrophoretic analysis of BSC extracts and the DTF. The upper well contained BSC extracts at a concentration of 8 mg of protein per ml and the lower well contained DTF at a concentration of 13 mg of protein per ml. The centre trough was filled with an absorbed rabbit anti-BSC serum.

found to inhibit the activity of proteases. The small molecular form of SCP was also present in the DTF when BSC was homogenized in chloroform-methanol (2:1) and then extracted with 0.1 M NaCl. This result indicates that the small form of SCP found in the DTF was not a product of brain protease action.

An additional experiment was carried out to ascertain the relative proportions of the dialyzable and non-dialyzable forms of SCP in BSC. It was reported by Lajtha and Marks (141) that brain proteases were inhibited by Cu^{++} ions. Accordingly, five 30 g samples of BSC were each homogenized at 0°C in 150 ml of 0.1 M NaCl containing 2 mM Cu^{++}, and held at various temperature for 16 hours. Aliquots of the supernatants were removed for the determination of total protein. The BSC extracts were then filtered through dialysis tubing and each DTF was concentrated tenfold by flash evaporation. The concentration of SCP in the BSC extracts and in each DTF was determined by the antibody-in-agar method of Mancini et al. (138) using a preparation of pure β_1-SCP (see following results) as standard. A standard calibration line is shown in Figure 10. The results of the quantitative immunodiffusion analyses are summarized in Table II. It will be seen that the presence of an enzyme inhibitor during homogenization had no noticeable effect on the amount of the smallest form of SCP in the DTF. This indicates that the smallest form of SCP is not derived from the large SCP by the action of brain proteases, but is probably present in the intact BSC.

Table II also illustrated that the highest amount of SCP as well as the highest yield of the dialyzable form of SCP were found in a BSC extract obtained from a spinal

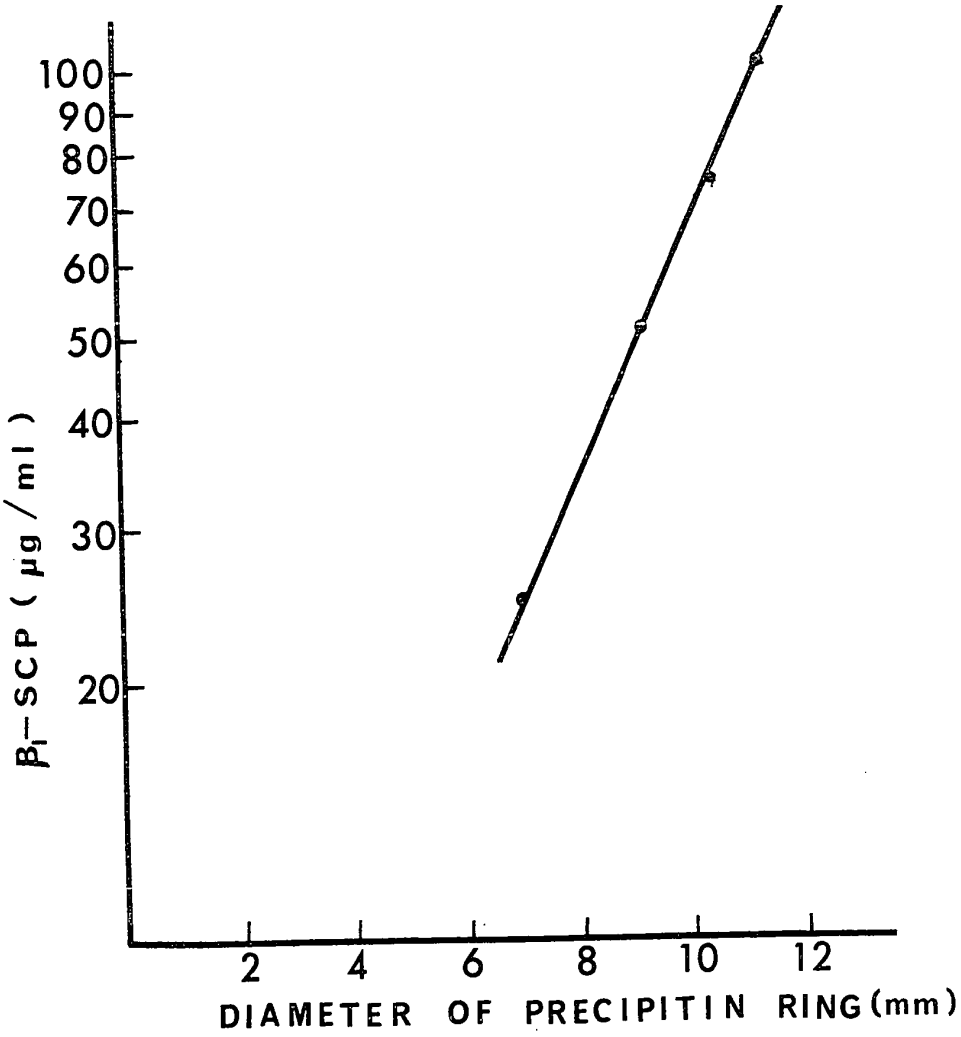


Figure 10. Relation between the area of the precipitates and the logarithm of the β_1-SCP concentration.

TABLE II

Effect of copper, duration and temperature of the extraction period on the yield of protein, SCP[a] and SCP-peptide in BSC extracts.[b]

Homogenizing medium	Duration and temperature of extraction period		BSC Extract		DTF of BSC Extract		Percent of SCP in peptide form
			Total protein	SCP	Total protein	SCP peptide	
	hrs.	$0^{o}C$	mg/ml	mg/ml x 10^{-2}	mg/ml	mg/ml x 10^{-2}	
0.1 M NaCl	16	-20	2.2	10.1 (4.5)[c]	0.25	0.75	
	16	4	2.5	7.5	0.33	0.40	
	5	37	2.0	5.0	0.18	0.35	6.6[d]
	16	-20					
	1	20	2.3	7.5	-	-	
0.1 M NaCl	16	-20	1.6	6.0	0.13	0.32	
	16	4	0.86	7.5	0.14	0.40	
2mM Cu^{++}	2	4	1.1	7.2	0.25	0.70	6.8[e]

a) Purified SCP, having a β_1-electrophoretic mobility was used as the standard for the determinations of SCP activity by the antibody-in-agar method (138).

b) Each extract was obtained from a homogenate of 30g. of bovine cord in 150 ml. of medium.

c) Figures in brackets indicate the percent of total protein in BSC extracts measured as SCP.

d) Average of three determinations of SCP peptide in absence of enzyme inhibitor.

e) Average of the three determinations of SCP peptide in presence of enzyme inhibitor.

cord homogenate that was frozen in plastic bags immediately after preparation, thawed within 10 minutes and centrifuged at 0°C. The lower yield of SCP obtained when the BSC extract was left overnight at 4°C might be attributed to enzymatic breakdown. It will be seen from Table II that SCP composed almost 5% of the total protein of spinal cord extracts and that the ratio of the non-dialyzable and dialyzable forms was approximately 15:1.

From Table II, it may be calculated that about 1% of the wet weight of BSC was obtained as protein by one extraction with 0.1 M NaCl. A total of 3% of the wet weight of BSC could be obtained as protein if the BSC was extracted with 0.1 M NaCl 18 times. However, it was found that most of the SCP was removed by two extractions although trace amounts of SCP could be detected after the fifth extraction.

THE NATURE OF BOVINE SCP

The heat stability of the bovine SCP was investigated by heating BSC extract at 100°C for 30 minutes (see "METHODS). After removing the precipitate, the supernatant was tested for the presence of SCP by double gel diffusion analyses with absorbed rabbit anti-D-BSCE serum. A precipitin line formed which fused with the line clearly visible opposite the unheated BSC extract. The result indicates that the antigen is heat resistant.

The SCP was also investigated for its stability at various pH. Aliquots of BSC extracts were dialyzed against 0.05 M glycine-HCl buffer, pH 2.0; Na-acetate-acetic

acid buffer, pH 4.0; Tris-HCl buffers, pH 6 and 8; and Na-borate-NaOH buffer, pH 10.0 for 16 hours. After removing the precipitates, the supernatants were tested for SCP activity by immunodiffusion and immunoelectrophoretic analyses. Results obtained indicated that SCP is stable at all these pHs.

ANALYSES OF OTHER MAMMALIAN SPINAL CORD EXTRACTS

Spinal cord extracts obtained from other mammals such as the human, rabbit, rat and mouse, were analyzed for the presence of SCP by the double diffusion in gel method using an absorbed anti-D-BSCE serum. The results are shown in Figure 11. The bovine, human, and rabbit spinal cord extracts contained 1 mg of protein per ml, while the concentrations of rat and mouse spinal cord extracts were 4 mg, and 11 mg protein per ml respectively. The continuous fused precipitin line indicates a reaction of immunological identity. It can be seen that the lines developed opposite the human and rabbit spinal cord extracts were as strong as that opposite the BSC extracts. When the rat, and mouse spinal cord extracts were tested at the 1 mg protein per ml level, only faint precipitin bands were observed. These results indicate either that the concentrations of SCP in mouse and rat spinal cord extracts are relatively low or that the non-antigenic portions of the SCP molecule in the rat and mouse differ widely from the non-antigenic part of bovine SCP.

ANALYSES OF OTHER BOVINE ORGANS FOR THE PRESENCE OF SCP

Bovine brain gray matter was carefully separated by blunt dissection from the

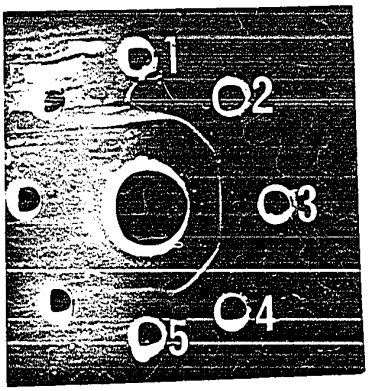


Figure 11. Double diffusion analysis of spinal cord extracts from various mammals.

The peripheral wells

(1) contained BSC extract at a concentration of 1 mg protein per ml

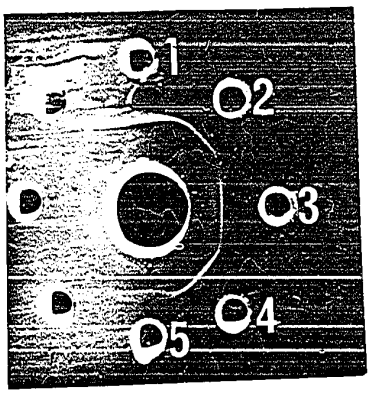
(2) contained human spinal cord extract at a concentration of 1 mg protein per ml.

(3) contained rabbit spinal cord extract at a concentration of 1 mg protein per ml.

(4) contained rat spinal cord extract at a concentration of 4 mg protein per ml.

(5) contained mouse spinal cord extract at a concentration of 11 mg protein per ml.

The centre well was filled with an absorbed rabbit anti-D-BSCE serum.



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The peripheral wells

(1) contained BSC extract at a concentration of 1 mg protein per ml

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(4) contained rat spinal cord extract at a concentration of 4 mg protein per ml.

(5) contained mouse spinal cord extract at a concentration of 11 mg protein per ml.

The centre well was filled with an absorbed rabbit anti-D-BSCE serum.

white matter and each tissue was extracted with 0.1 M NaCl. Bovine liver, adrenal, and peripheral nerve as well as fetal calf brain and adult bovine brain were extracted with 0.1 M NaCl. Extracts of these tissues (except that of peripheral nerve), tested at a concentration of 1 mg protein per ml in immunodiffusion analyses using an absorbed rabbit anti-D-BSCE serum were negative. When the extracts were retested at concentrations of 20 to 30 mg protein per ml some were positive. The results are presented in Figure 12. It can be seen that the antiserum produced with spinal cord extracts detected several species-specific antigens as shown by the precipitin bands formed opposite other bovine organ extracts. One of the lines developed opposite the brain white matter extract fused with the line formed opposite the spinal cord extract, indicating a reaction of identity. This was identified as the line formed by SCP. However, the concentration of SCP in brain white matter extract was relatively very low, because the precipitin band was faint and quite close to the antigen well. No SCP precipitin band was observed opposite concentrated extracts of brain gray matter, liver, adrenal, fetal brain, and adult brain extracts. It is interesting to note that SCP was present in the saline extracts of peripheral nerve in roughly the same concentration as in bovine cord. The above extracts were retested using monospecific anti-β_1-SCP serum when it became available later. The result is shown in Figure 13. A continuous precipitin line formed opposite BSC, peripheral nerve and brain white matter extracts, indicating a reaction of identity. The line opposite the brain white matter extract is much fainter than the line opposite the spinal cord and peripheral nerve extracts. This result indicates that the brain white matter extract contained relatively much less SCP than spinal cord. No precipitin line

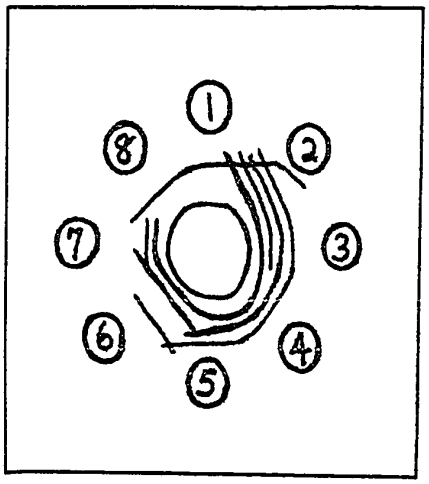

Figure 12. Double diffusion analysis of 0.1 M NaCl extracts of bovine organs.

(1) BSC extract (1 mg protein per ml).

(2) Bovine brain white matter extract (25 mg protein per ml).

(3) Bovine brain gray matter extract (25 mg protein per ml).

(4) Bovine whole brain extract (20 mg protein per ml).

(5) Fetal calf brain extract (20 mg protein per ml).

(6) Bovine liver extract (30 mg protein per ml).

(7) Bovine adrenal extract (20 mg protein per ml).

(8) Bovine peripheral nerve extract (1 mg protein per ml).

The centre well was filled with an absorbed rabbit anti-D-BSCE serum.

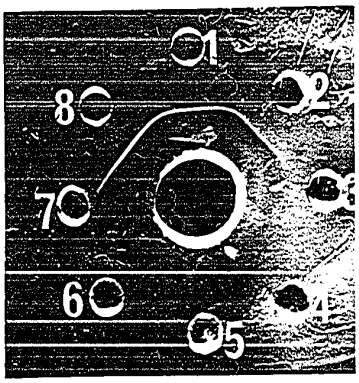

Figure 13. Double diffusion analysis of bovine organ extracts developed with a rabbit anti-β_1-SCP serum.

Peripheral wells

(1) BSC extract (1 mg protein per ml).

(2) Bovine brain white matter extract (25 mg protein per ml).

(3) Bovine brain gray matter extract (25 mg protein per ml).

(4) Bovine whole brain extract (20 mg protein per ml).

(5) Fetal calf brain extract (20 mg protein per ml).

(6) Bovine liver extract (30 mg protein per ml).

(7) Bovine adrenal extract (20 mg protein per ml).

(8) Bovine peripheral nerve extract (1 mg protein per ml).

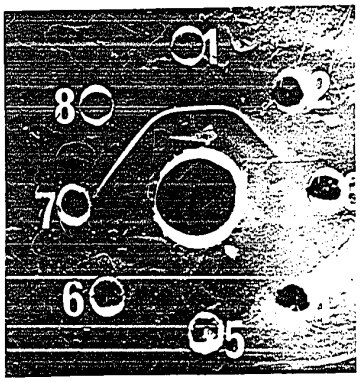

Figure 13. Double diffusion analysis of bovine organ extracts developed with a rabbit anti-β_1-SCP serum.

Peripheral wells

(1) BSC extract (1 mg protein per ml).

(2) Bovine brain white matter extract (25 mg protein per ml).

(3) Bovine brain gray matter extract (25 mg protein per ml).

(4) Bovine whole brain extract (20 mg protein per ml).

(5) Fetal calf brain extract (20 mg protein per ml).

(6) Bovine liver extract (30 mg protein per ml).

(7) Bovine adrenal extract (20 mg protein per ml).

(8) Bovine peripheral nerve extract (1 mg protein per ml).

was observed opposite other organ extracts. Thus, it appears that SCP is an organ-specific antigen which is found only in nervous tissue and is largely restricted to the spinal cord in the central nervous system.

The results of the immunoelectrophoretic analyses of extracts of bovine peripheral nerve and rat spinal cord are shown in Figure 14. The pattern was developed with an absorbed anti-D-BSCE serum. It can be seen that the SCP in these extracts also occurs in two molecular forms with different electrophoretic mobilities as both profiles contain double arcs.

Since SCP is a nervous tissue antigen, it was of interest to ascertain whether it was antigenically similar to the encephalitogenic basic protein of the nervous system. The result of a comparison of the bovine basic protein and purified SCP by immunodiffusion analysis is shown in Figure 15. The antiserum used was a rabbit anti-β_1-SCP serum. No precipitin line was developed opposite the basic protein at concentrations from 0.2 mg to 5 mg protein per ml. This indicates that SCP and basic protein do not contain the same antigenic groups.

LOCATION OF SCP IN SUBCELLULAR FRACTIONS OF BOVINE SPINAL CORD

The four usual subcellular fractions, namely crude nuclear, mitochondrial, microsomal and soluble fractions, were prepared from homogenates of BSC in 0.32 M sucrose by differential centrifugation (see METHODS). The pellets were extracted with 0.1 M NaCl and were analyzed by immunodiffusion tests for SCP using an absorbed anti-D-BSCE serum. The antigen was detected only in the soluble fraction.

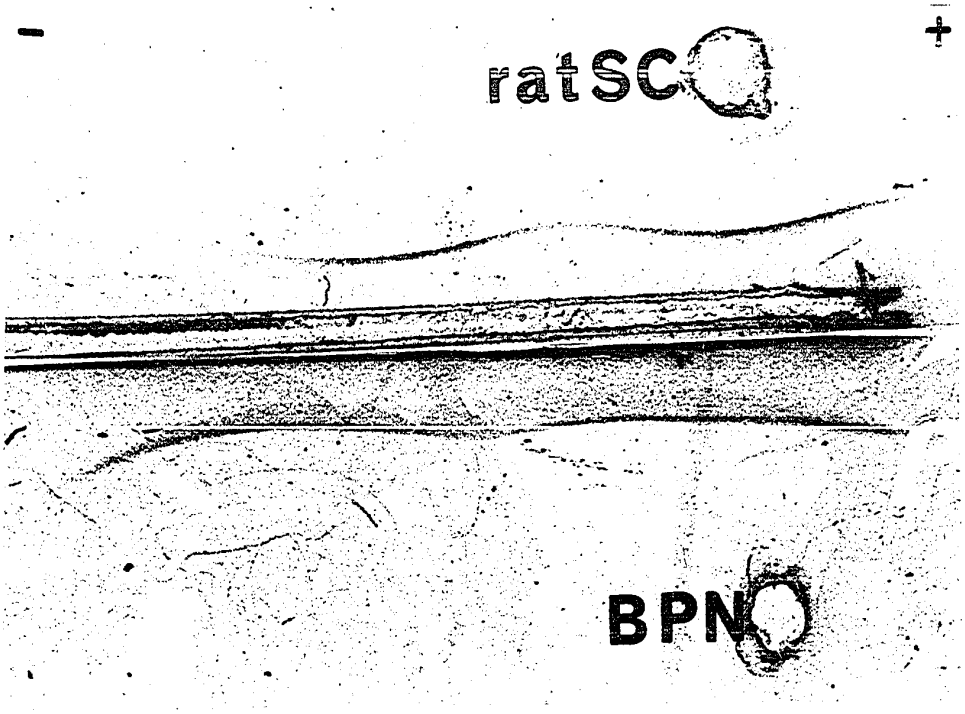

Figure 14. Immunoelectrophoretic analysis of extracts or rat spinal cord, 7.8 mg protein per ml (top), and of bovine peripheral nerve 4.5 mg protein per ml (bottom), developed with an absorbed rabbit anti-D-BSCE serum.

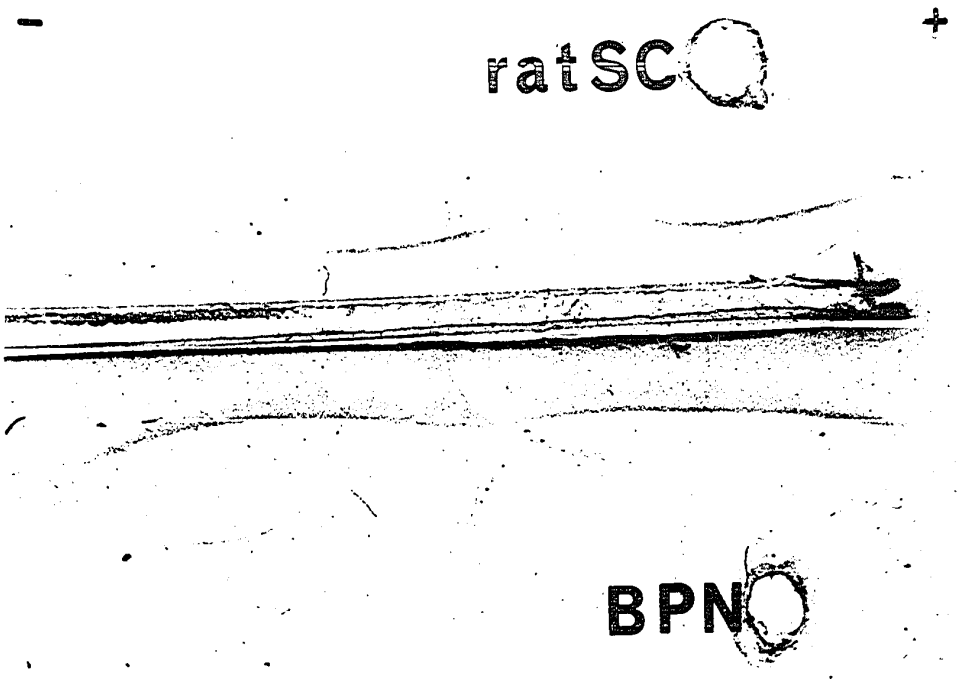

Figure 14. Immunoelectrophoretic analysis of extracts or rat spinal cord, 7.8 mg protein per ml (top), and of bovine peripheral nerve 4.5 mg protein per ml (bottom), developed with an absorbed rabbit anti-D-BSCE serum.

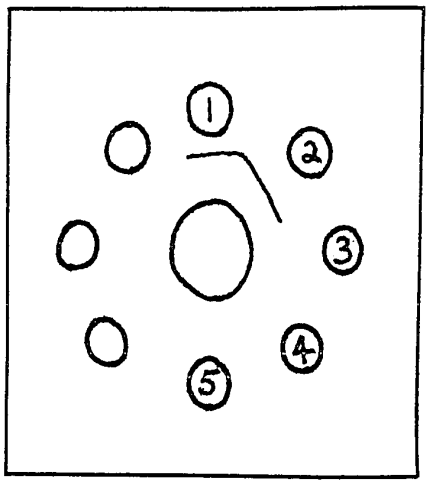

Figure 15. Immunodiffusion analysis of bovine encephalitogenic protein (BEP)* and purified β_1-SCP.

The peripheral wells were filled with

(1) BSC extract (1 mg protein per ml).

(2) β_1-SCP (0.2 mg protein per ml).

(3) Bovine encephalitogenic protein (0.2 mg protein per ml).

(4) BEP (1 mg protein per ml).

(5) BEP (5 mg protein per ml).

The centre well was filled with rabbit anti-β_1-SCP serum.

* BEP was a gift of Dr. M. Kies of N.I.H., Bethesda, Maryland.

LOCATION OF SCP IN SPINAL CORD

As mentioned above, SCP was not detected in concentrated extracts of the gray matter of bovine brain, but was located in concentrated extracts of the white matter. It was of interest to find out whether the same distribution of SCP also occurred in the spinal cord. Bovine cord white matter was carefully separated from the gray matter and each tissue was minced and extracted with 0.1 M NaCl. After removing the precipitate, the supernatant was tested by immunodiffusion analysis for SCP. The result is shown in Figure 16. No precipitin line was formed opposite the spinal cord gray matter when tested at a concentration of 1 mg and 3 mg protein per ml. This indicated that SCP is not present or is present in very low concentration in the gray matter of spinal cord.

DETECTION OF SCP IN THE COURSE OF DEVELOPMENT

Since SCP was found chiefly in the white matter of spinal cord, it was of interest to determine at what stage in development it first appeared. Rats were used for this investigation. Pregnant rats were obtained from the breeding farm a few days prior to time of parturition. Spinal cords were removed from new born, 5-, 8-, 9- and 10-day old rats. The cords were homogenized and extracted with 0.1 M NaCl, and the concentrated extracts tested for SCP by immunodiffusion analyses. The results are shown in Figure 17. Precipitin bands can be seen opposite the 9- and 10-day old rat spinal cord extracts. SCP was not detected in new born, 5- and 8-day old rat spinal cord extracts at the concentrations tested. The antiserum used for this detection was an absorbed rabbit anti-D-BSCE serum since it was shown that rat SCP cross-reacted with bovine SCP. In order to obtain a precipitin line indicating the presence of SCP, the extracts of

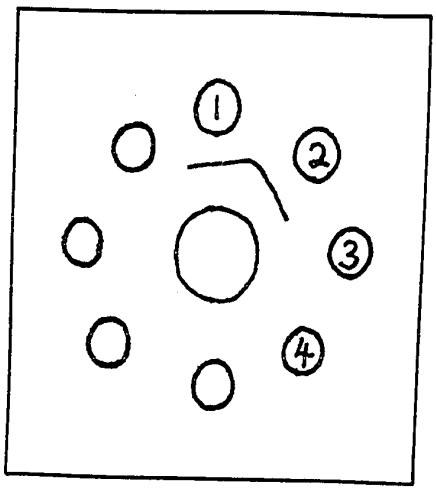

Figure 16. Double diffusion analysis of extracts of white and gray matter of bovine spinal cord.

The peripheral wells were filled with

(1) BSC extract (1 mg protein per ml).

(2) White matter extract of bovine spinal cord (1 mg protein per ml).

(3) Gray matter extract of bovine spinal cord (1 mg protein per ml).

(4) Gray matter extract of bovine spinal cord (3 mg protein per ml).

The centre well was filled with an absorbed rabbit anti-D-BSCE serum.

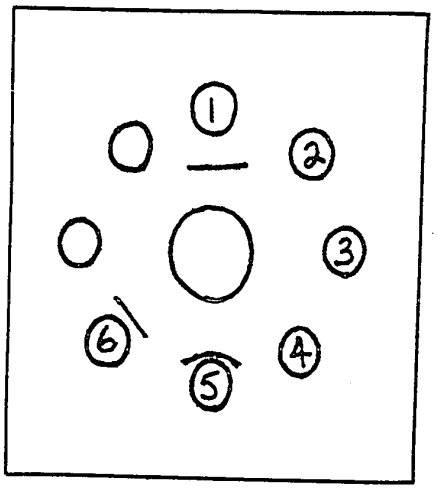

Figure 17. Double diffusion analysis of rat spinal cord extracts at various stages of development.

Peripheral wells were filled with

(1) Adult rat spinal cord extract (4 mg protein per ml).

(2) New born rat spinal cord extract (7 mg protein per ml).

(3) 5 day-old rat spinal cord extract (12 mg protein per ml).

(4) 8 day-old rat spinal cord extract (14 mg protein per ml).

(5) 9 day-old rat spinal cord extract (16 mg protein per ml).

(6) 10 day-old rat spinal cord extract (19 mg protein per ml).

The centre well was filled with an absorbed rabbit anti-D-BSCE serum.

immature rat spinal cords had to be concentrated to more than 10 mg protein per ml. The identification of SCP in the 9-day old rat spinal cord extracts indicated that SCP is synthesized before the onset of myelination.

CHROMATOGRAPHY OF SPINAL CORD EXTRACT

In the early stages of this study, an attempt was made to separate the three forms of SCP from other cord proteins by gel filtration chromatography on Sephadex G-200. The elution diagram is shown in Figure 18. Four to five poorly resolved peaks were obtained. SCP activity was found in fractions 6 to 20. These fractions also contained serum proteins. Obviously, using gel-filtration to separate the three forms of SCP at this stage of purification was not practical.

In view of the neutral and basic nature of the forms of SCP, a cation exchanger was chosen for the initial separation of the three forms of SCP.

BATCH ABSORPTION OF BSC EXTRACT ON DEAE-SEPHADEX A-50

It was found that many of the undesirable anionic components of BSC extracts could be removed by batch-wise absorption with DEAE-Sephadex A-50 equilibrated with 0.05 M Tris-HCl, pH 7.0. The absorbed BSC extract was then concentrated by flash evaporation to 25 mg protein per ml and desalted on a column of Sephadex G-25 with 0.05 M sodium acetate buffer, pH 5.5.

It was noted that 90% of the protein in BSC extracts is removed after absorption

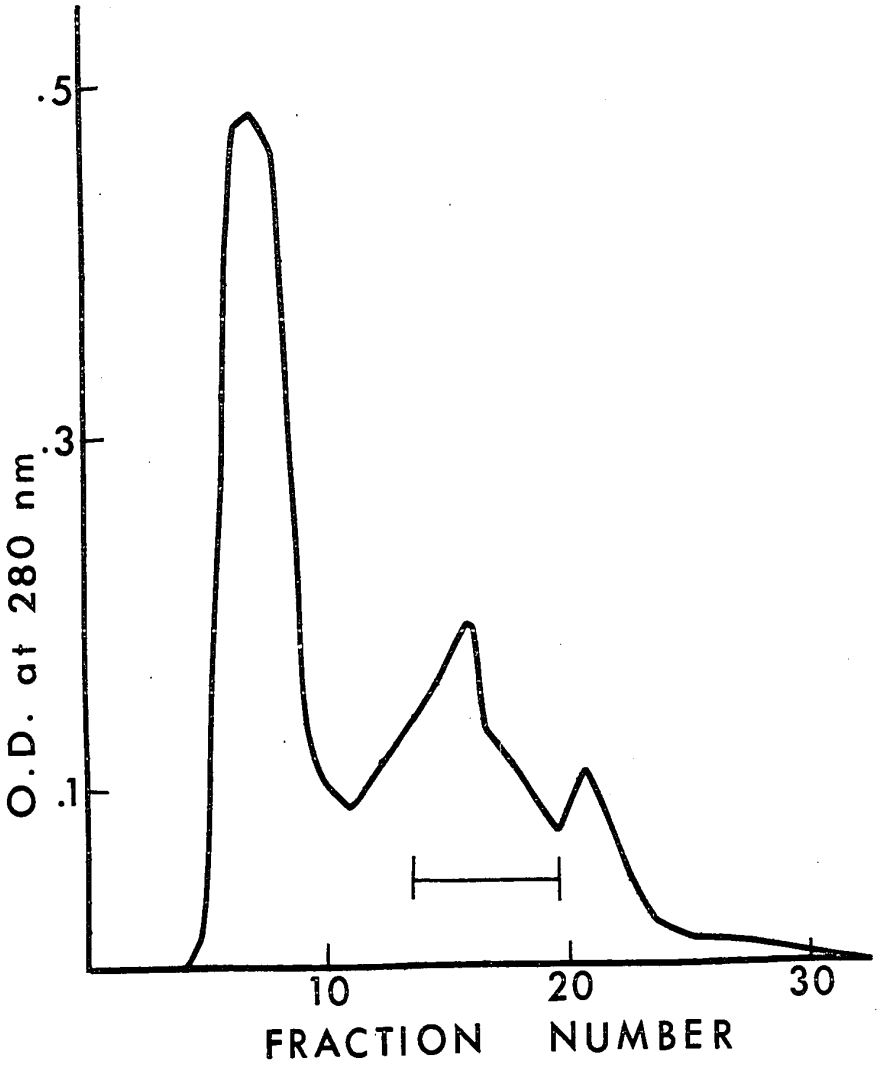

Figure 18. Elution pattern of BSC extracts on Sephadex G-200

⊢—⊣ indicates fractions containing SCP activity. The proteins were eluted with 0.1M NaCl and 5 ml fractions were collected at 30 minute intervals.

with DEAE-Sephadex A-50, and that this figure represents 0.1% of the weight of the starting material, BSC. By the antibody-in-agar method of Mancini et al. (138), it was found that after absorption with DEAE-Sephadex A-50, a BSC extract (0.2 mg protein per ml) contained 0.05 mg SCP protein per ml. Similarly, unabsorbed BSC extracts (0.5 mg protein per ml) contained 0.015 mg SCP protein per ml. As 10% of the total protein is recovered from BSC extract after absorption with DEAE-Sephadex A-50 one can calculate that SCP is not removed by this treatment.

CHROMATOGRAPHY OF DEAE ABSORBED BSC EXTRACT ON CM-52 CELLULOSE

The DEAE absorbed BSC extract was equilibrated with 0.05 M sodium acetate buffer, pH 5.5 and then applied to a column of CM-52 cellulose. The proteins were eluted with 0.05 M sodium-acetate buffer, pH 5.5, containing stepwise gradients of NaCl. A typical elution pattern composed of 5 peaks is shown in Figure 19. The material in each tube was analyzed by immunodiffusion and immunoelectrophoresis to locate SCP using an absorbed rabbit anti-D-BSCE serum. The first peak was found to contain serum proteins. The second peak contained the dialyzable form of SCP having the γ-globulin electrophoretic mobility as well as serum proteins. The immunoelectrophoretic pattern of peak 2 is shown in Figure 20. Peaks 3 and 4 contained serum proteins and a trace amount of SCP. The shoulder on the ascending limb of peak 5 (dotted area) and the cross-hatched area on the descending limb of peak 5 contained the larger molecular forms of SCP having the electrophoretic mobilities of β_1 and γ-globulins respectively. The result of the immunoelectrophoretic analyses is presented in Figure 21.

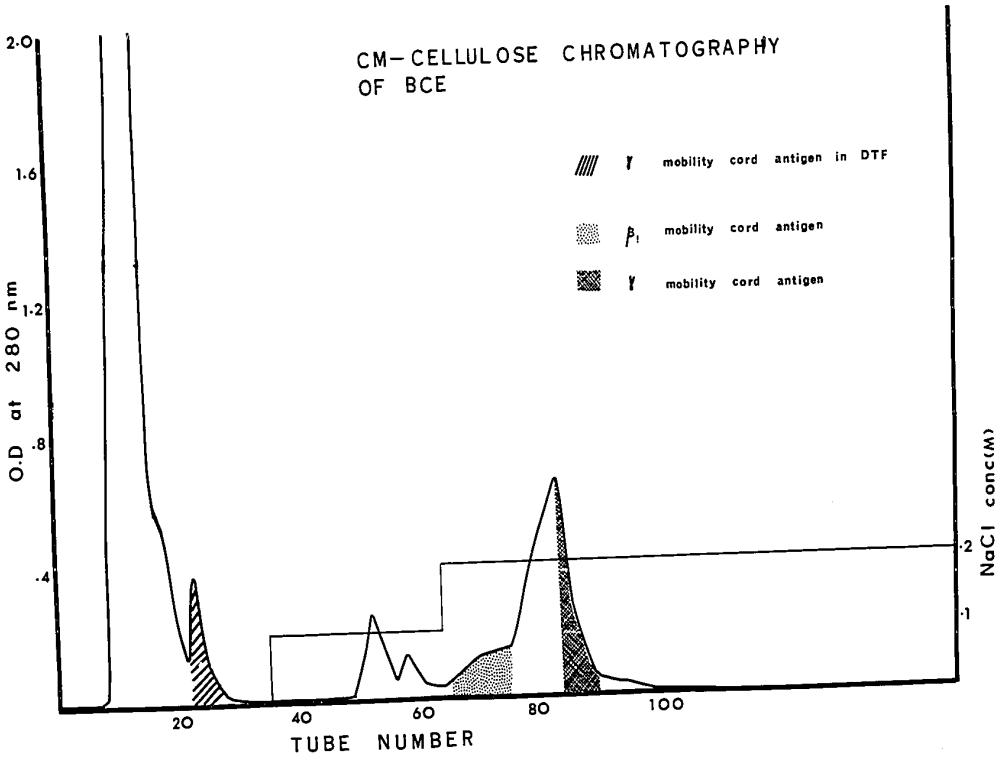

Figure 19. A CM-52 cellulose chromatogram of DEAE absorbed BSC extracts. The proteins were eluted with 0.05M sodium acetate buffer, pH 5.5, and a stepwise gradient of sodium chloride. The 5 ml fractions were collected every 24 minutes.

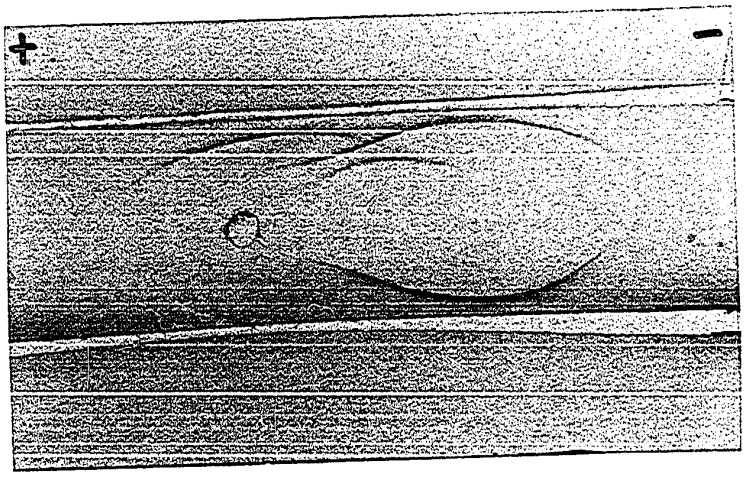

Figure 20. Immunoelectrophoretic analysis of peak 2 from CM-cellulose chromatography of DEAE-Sephadex A-50 absorbed BSC extracts.

Centre well contained material from peak 2.

Upper trough contained unabsorbed rabbit anti-D-BSCE serum.

Lower trough contained absorbed rabbit anti-D-BSCE serum.

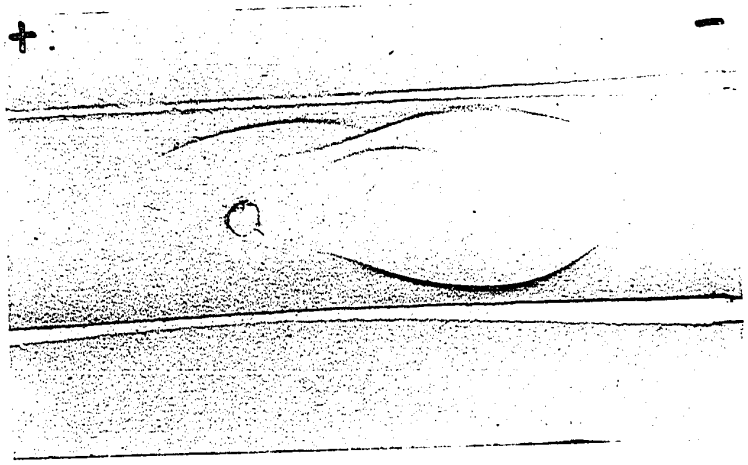

Figure 20. Immunoelectrophoretic analysis of peak 2 from CM-cellulose chromatography of DEAE-Sephadex A-50 absorbed BSC extracts.

Centre well contained material from peak 2.

Upper trough contained unabsorbed rabbit anti-D-BSCE serum.

Lower trough contained absorbed rabbit anti-D-BSCE serum.

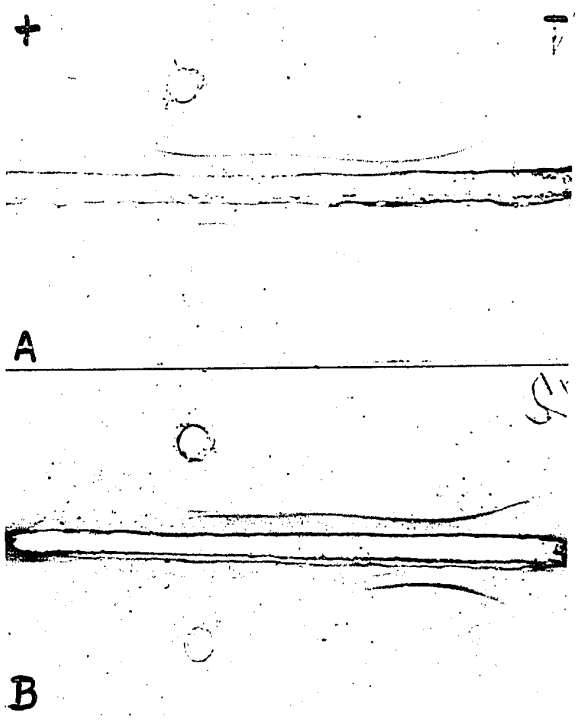

Figure 21. Immunoelectrophoretic analyses of materials from ascending and descending limbs of peak 5 from CM-cellulose chromatography of DEAE absorbed BSC extracts.

A. Upper well contained DEAE absorbed BSC extracts.

Lower well contained material from ascending limb of peak 5.

B. Upper well contained DEAE absorbed BSC extracts.

Lower well contained material from descending limb of peak 5.

In A and B, the centre troughs were filled with an absorbed rabbit anti-D-BSCE serum.

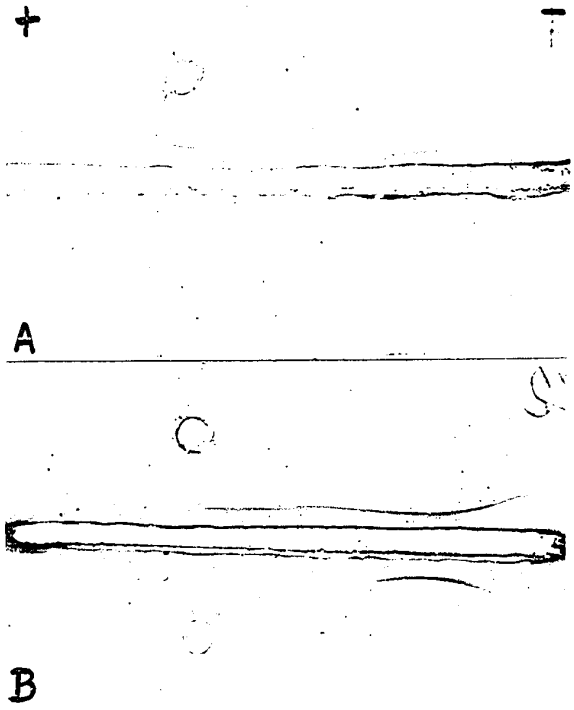

Figure 21. Immunoelectrophoretic analyses of materials from ascending and descending limbs of peak 5 from CM-cellulose chromatography of DEAE absorbed BSC extracts.

A. Upper well contained DEAE absorbed BSC extracts.

Lower well contained material from ascending limb of peak 5.

B. Upper well contained DEAE absorbed BSC extracts.

Lower well contained material from descending limb of peak 5.

In A and B, the centre troughs were filled with an absorbed rabbit anti-D-BSCE serum.

The fraction containing the SCP with the ß1-globulin electrophoretic mobility was found to be free of serum contaminants by immunodiffusion analysis with a rabbit anti-bovine serum . The fraction containing the larger form of the SCP having a γ-globulin electrophoretic mobility was found to be contaminated with small amount of bovine γ-globulin by immunodiffusion analysis.

ANALYTICAL POLYACRYLAMIDE GEL ELECTROPHORESIS

The BSC extract was analysed by polyacrylamide gel electrophoresis at pH 4.3. Only one band was located 4.5 cm from the anodic end (Figure 22). Obviously, running the gel at an acidic pH was not useful because no separation of the component proteins (mostly globulins) was achieved.

The BSC extract was then analyzed by polyacrylamide gel electrophoresis at pH 8.9. The result is presented in Figure 23, 1. At least 10 bands could be counted. When the DEAE absorbed BSC extract was subjected to polyacrylamide gel electrophoresis at pH 8.9, one could see from Figure 23, 2, that a number of acidic proteins had been removed.

The location of the position of the SCP after electrophoresis of BSC extract in polyacrylamide gel was determined as follows: when the run was completed, the gel was placed in a trough in a dish containing solidified agar (see METHODS) and the antigen was located by the position of the immunodiffusion arc that formed between the gel and a trough containing an absorbed anti-D-BSCE serum. When the disc gel was run until the tracking dye travelled 6 cm, the precipitin arc was 4 cm. long. The mid-point of

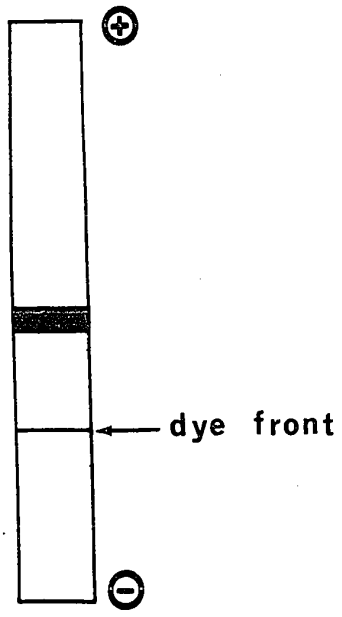

Figure 22. Polyacrylamide disc gel electrophoresis of BSC extracts at pH 4.3

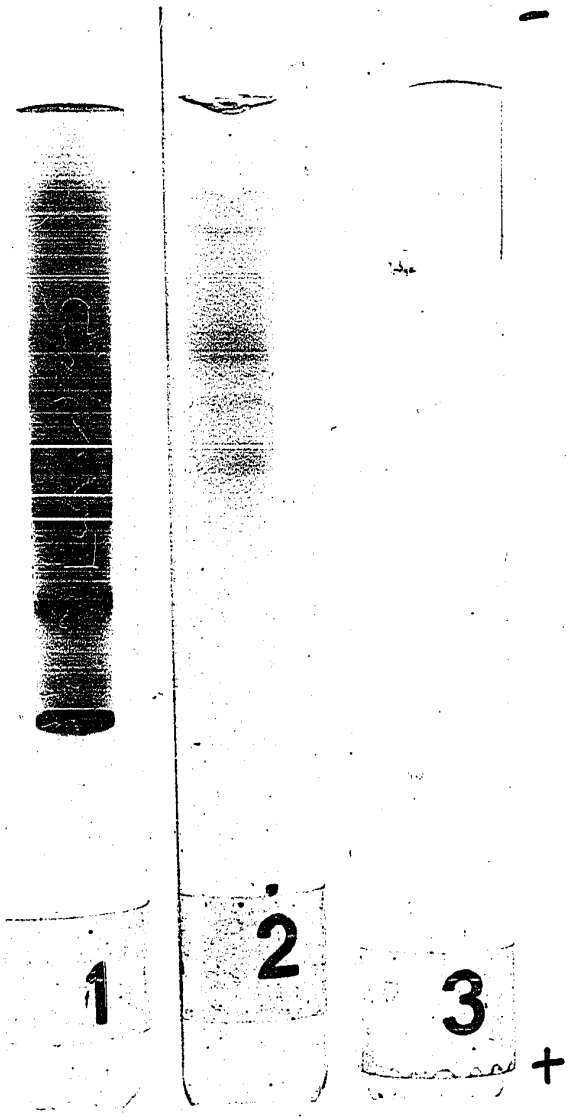

Figure 23. Analytical polyacrylamide gel electrophoretogram of (1) BSC extracts, 250 µg; (2) BSC extracts after absorption with DEAE-Sephadex A-50, 150 µg; (3) purified form of SCP of β_1-globulin electrophoretic mobility eluted from CM-cellulose in the ascending limb of peak 5. (see Figure 19).

the arc was about 1 cm from the cathode end of the gel (Figure 24). Since only one arc was formed, this would indicate that the three forms of SCP migrate to similar positions in polyacrylamide gel.

The material from the ascending shoulder of peak 5 of the CM-cellulose chromatogram of DEAE absorbed BSC extract was found to be free of serum proteins by immunodiffusion analyses and was also shown to contain the SCP of β_1-globulin electrophoretic mobility by immunoelectrophoretic analyses. When this fraction was subjected to analysis by polyacrylamide gel electrophoresis, only one band was formed at pH 8.9. The result is presented in Figure 23, 3. When one of the unstained gel was analyzed by immunodiffusion experiment, one arc was noted, the mid-point of which was 1.0 cm from the cathodic end of the gel (Figure 25). Thus, the β_1-SCP obtained from CM-52 cellulose chromatography is electrophoretically homogeneous and immunologically pure.

MOLECULAR WEIGHT DETERMINATION OF SCP BY GEL FILTRATION

A Sephadex G-100 column was calibrated with the proteins listed in Table III for the molecular weight estimation of the three forms of SCP. The elution volumes, Ve, of these proteins were determined as described under "Methods". The elution volumes were then plotted on a graph against the logarithms of the molecular weights of the proteins (Figure 26). The graph was linear over the molecular weight range of 12,000 -85,000 but the useful working range of Sephadex G-100 extends to approximately 150,000. The elution volume for the pure β_1-SCP (obtained from the ascending shoulder

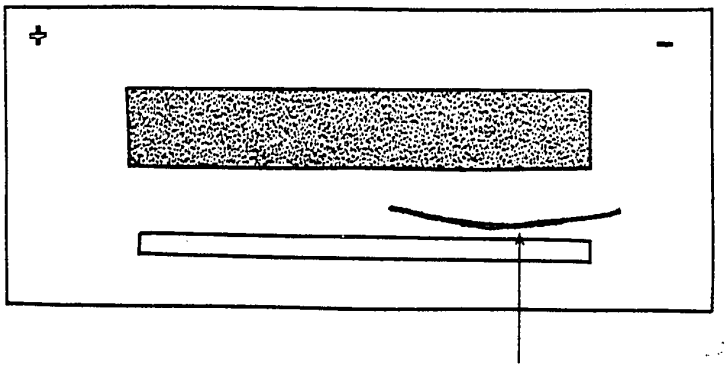

Figure 24. Immunodiffusion analysis of an unstained polyacrylamide gel following electrophoresis of BSC extracts at pH 8.9.

The trough was filled with an absorbed rabbit anti-D-BSCE serum.

 polyacrylamide gel.

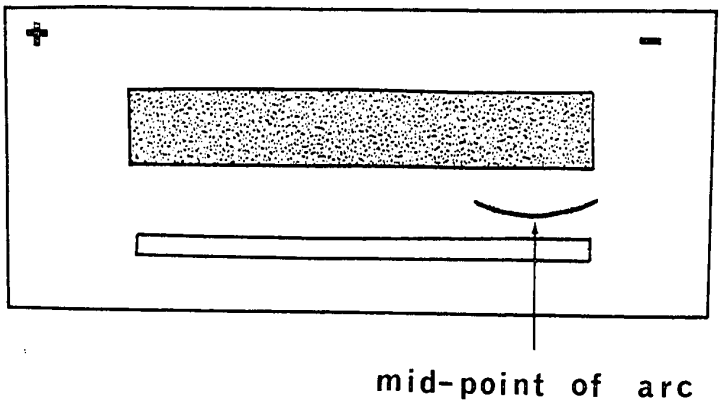

Figure 25. Immunodiffusion analysis of an unstained polyacrylamide gel following electrophoresis of the purified form of SCP of β_1-globulin electrophoretic mobility eluted from CM-52 cellulose in the ascending limb of peak 5. (see Figure 19).

The trough was filled with an unabsorbed rabbit anti-D-BSCE serum.

polyacrylamide gel.

TABLE III

Elution volumes of proteins of known molecular weight and purified forms of SCP on a Sephadex G-100 column

Proteins	Molecular Weight	Ve (ml)
Cytochrome c	12,400	92
Ribonuclease	13,700	88
α-chymotrypsinogen A (Type II)	25,000	78
Bovine Serum Albumin	67,000	56
Bovine γ-globulin	160,000	44
Blue Dextran	2,000,000	40
β_1-SCP	18,000*	84
γ-SCP	12,400*	92
γ-SCP (DTF)	4,500*	112

* Calculated from graph on Figure 26.

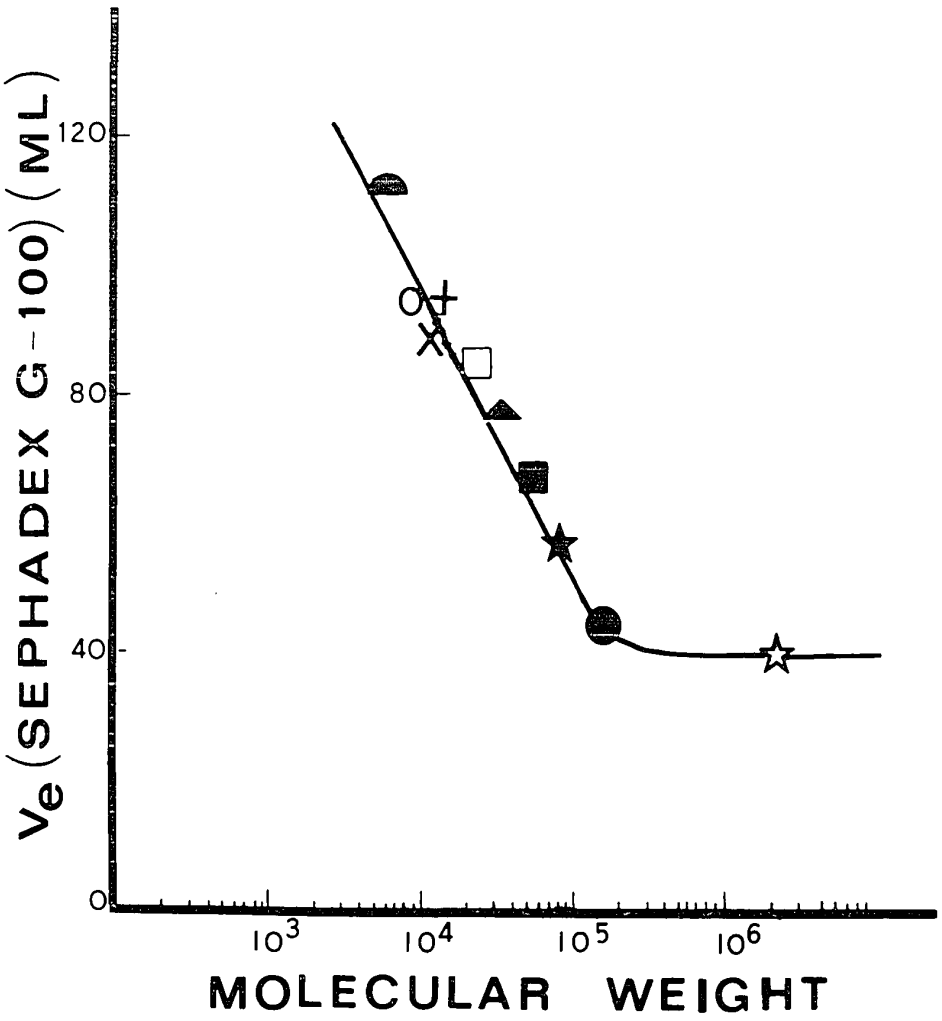

Figure 26. Plot of elution volume (Ve) against log (molecular weight) for proteins on a Sephadex G-100 column. ☆ Blue Dextran; ● , Bovine γ-globulin; ★ Bovine serum albumin; ■ Crystalline egg albumin; ▲ α-chymotrypsinogen A; □ β_1-electrophoretic mobility SCP; X , Ribonuclease; +, Cytochrome c; O , γ-electrophoretic mobility SCP; ◠ , γ-electrophoretic mobility SCP in DTF.

of peak 5 of CM-52 cellulose chromatography (see Figure 19) was calculated to be 84 ml. This elution volume was found to correspond to a molecular weight of 18,000. The elution volume for the larger form of γ-SCP (using material obtained from the descending limb of peak 5 of the CM-52 cellulose chromatogram) was calculated to be 92 cc. The antigen was detected in the column effluents by immunodiffusion analyses. The Ve was found to correspond to a molecular weight of 12,400 when the calibration curve for the column given in Figure 26 was used. A concentrated solution of the DTF of a BSC extract was used for the estimation of the molecular size of the dialysable form of the antigen having a γ-globulin electrophoretic mobility. The elution volume of this material was calculated to be 112 ml which corresponded to a molecular weight of 4,500.

MOLECULAR WEIGHT DETERMINATION OF β_1-SCP BY DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

The molecular weight of the pure β_1-SCP obtained by CM-52 cellulose chromatography was also determined by dodecyl sulfate polyacrylamide gel electrophoresis. The calibration curve (Figure 27), was obtained by calculating the mobilities in polyacrylamide gels of the proteins of known molecular weights listed in Table III. The mobility of each protein was calculated using the formula described under "Methods". After destaining, the protein band of β_1-SCP was found to be 4.1 cm away from the cathodic end and the mobility was calculated to be 0.704. This value was found to correspond to a molecular weight of 19,500, on the calibration plot in Figure 27. This

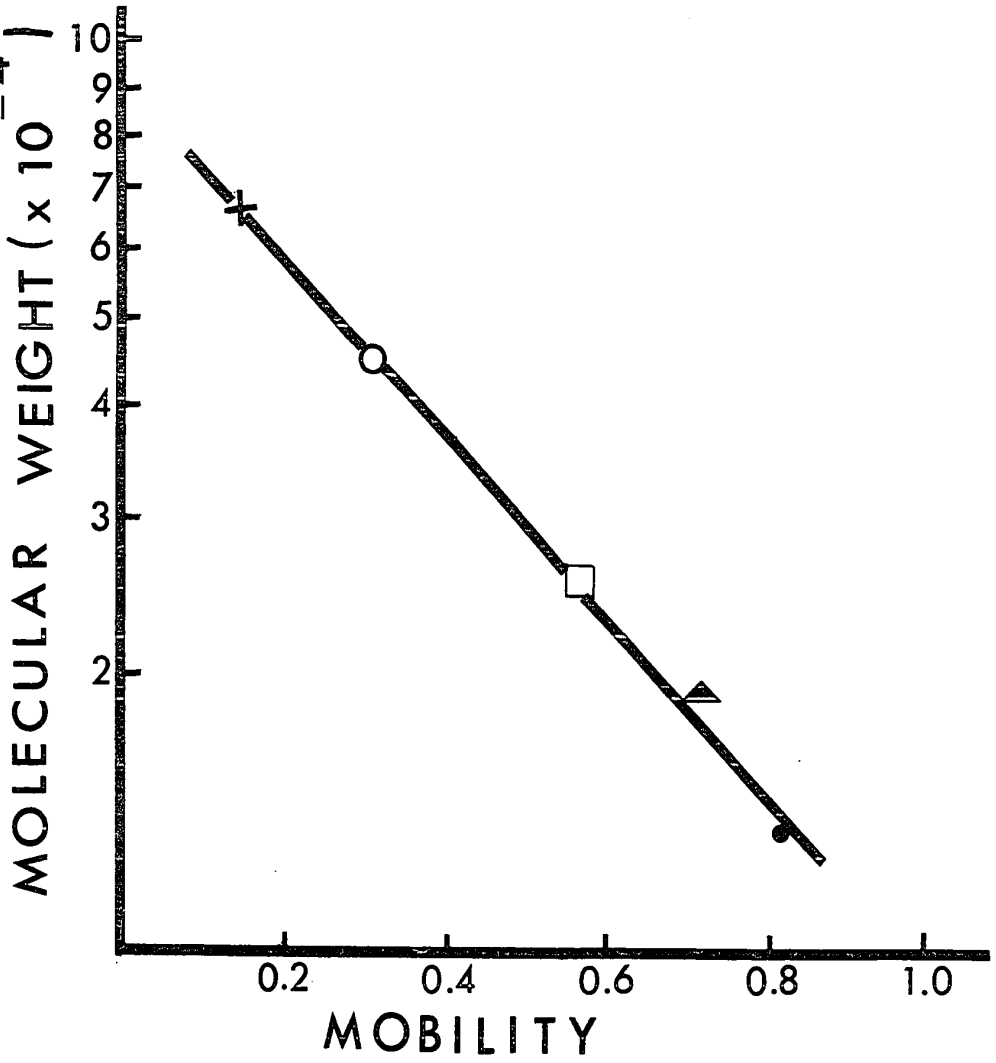

Figure 27. Plot of mobilities of proteins in SDS-polyacrylamide gels against the log of their molecular weights ● , Ribonuclease; + , Bovine serum albumin; ○ , Ovalbumin; □ , α-chymotrypsinogen A; ▲ , β_1-SCP.

figure agrees reasonably well with the molecular weight as estimated by gel filtration.

AMINO ACID ANALYSIS OF β_1-SCP

The amino acid analysis of β_1-SCP was determined in a Beckman 120B automatic amino acid analyzer (see METHODS). The number of micromols of each amino acid was converted to mols/100 mols of amino acid. No correction was made for hydrolytic losses and the value for ammonia was not included. The amino acid composition of β_1-SCP is presented in Table IV.

The pure antigen contained 21% of the dicarboxylic acids, such as aspartic acid and glutamic acid. The basic amino acids composed 16%. The minimum number of residues was calculated using the value of histidine as 1 residue. On this basis, the minimum number of residues in β_1-SCP was found to be 64. The minimum molecular weight was calculated to be 6,913 from the minimum number of amino acid residues. The molecular weight of β_1-SCP was estimated to be 18,000 using a calibrated Sephadex G-100 column (Figure 26). The total number of residues in β_1-SCP is thus 3 times the calculated minimum value. For example, there are 3 residues of histidines and 18 residues of glycine. No cystine was detected.

CHROMATOGRAPHY OF BSC EXTRACT ON CM-SEPHADEX C-25

In later experiments, BSC was extracted with 0.05 M sodium acetate-0.05M NaCl buffer, pH 5.5. The yield of SCP was comparable to that obtained by extraction

TABLE IV

AMINO ACID COMPOSITION OF β_1-SCP *

Amino Acid	Composition	Minimum No. of Residues
	mols/100 mols amino acid	
Lysine	10.0	6
Histidine	1.5	1
Arginine	5.1	3
Aspartic acid	10.1	6
Threonine	7.3	5
Serine	6.6	4
Glutamic acid	10.9	7
Proline	4.0	3
Glycine	8.7	6
Alanine	6.7	4
Half cystine	0.0	-
Valine	7.7	5
Methionine	1.9	1
Isoleucine	4.3	3
Leucine	8.9	6
Tyrosine	2.0	1
Phenylalanine	3.8	3

* The presence of carbohydrate or lipid was not determined.

with 0.1 M NaCl. The BSC extract was concentrated to 10 mg protein per ml in dialysis tubing and then stirred into a suspension of CM-Sephadex C-25 that had been equilibrated with 0.05 M sodium acetate—0.05 M NaCl buffer pH 5.5. The protein to ion-exchanger ratio was 1:10. The mixture was stirred at room temperature for one hour and the supernatant was filtered off. The wet gel was then transferred to a column (60 cm x 5 cm) and was washed with buffer until the O.D. at 280 nm of the effluent was 0. The absorbed proteins were then eluted with a stepwise gradient of NaCl in the pH 5.5 buffer. The elution profile is shown in Figure 28. The SCP activity was detected in every peak. By immunoelectrophoretic analyses, γ-SCP was detected in the peak eluted with 0.4 M NaCl. It was also found to be free of serum contaminants by immunodiffusion analyses. When this fraction was subjected to analysis by polyacrylamide gel electrophoresis, at pH 8.9, only one band was formed (Figure 29).

CHROMATOGRAPHY OF DTF OF BSC EXTRACT ON CM-SEPHADEX C-25

Recently, BSC extract was obtained by homogenizing BSC with 0.05 M sodium acetate-.05M NaCl buffer, pH 5.5. The DTF obtained from the BSC extract was not concentrated by flash evaporation and normally had a protein content of 0.2-0.3 mg protein per ml. The DTF, containing 2,000 mg protein was adjusted to pH 4.5 with acetic acid and was then added to a slurry of 20 g of CM-Sephadex C-25 that had been equilibrated with 0.05 M sodium acetate -.05 M NaCl buffer, pH 4.5. The protein to gel ratio was 1:10. The mixture was stirred for one hour at room temperature. Then the supernatant was filtered off and the wet gel was packed into a column measuring

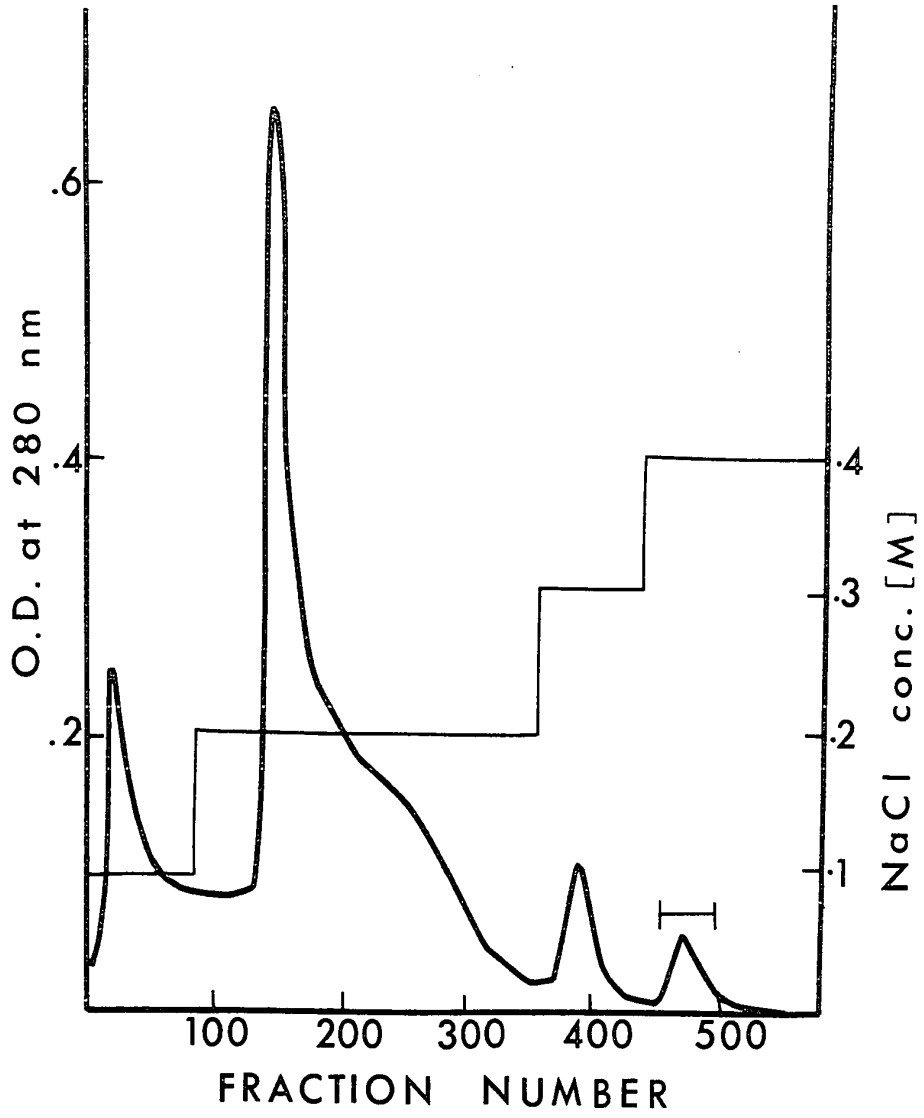

Figure 28. CM-Sephadex C-25 chromatogram of BSC extracts. The proteins were eluted with a stepwise gradient of NaCl in 0.05M sodium acetate-0.05M NaCl buffer, pH 5.5. The fraction volume was 10 ml and was collected at a rate of 60 ml per hour.

⊢⊣ indicates the purified form of SCP with γ electrophoretic mobility.

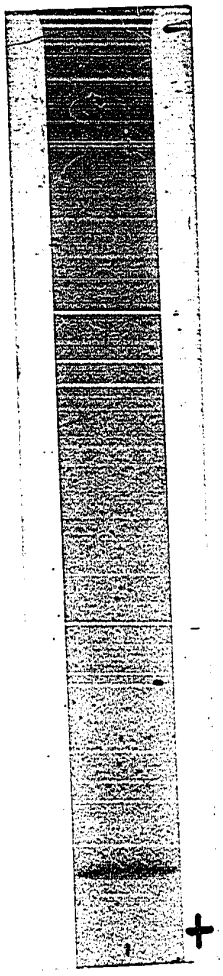

Figure 29. Analytical polyacrylamide gel electrophoretogram of purified form of SCP of γ electrophoretic mobility eluted from CM-Sephadex C-25. (see Figure 28). The lower band represents the dye marker.

60 cm x 5 cm. The absorbed proteins were eluted by a stepwise gradient of NaCl in the pH 4.5 buffer. The elution profile is shown in Figure 30. The SCP was detected in fractions 85 to 120 which had been eluted with NaCl concentrations between 0.25-0.4 M. The SCP activity was detected by immunodiffusion analyses using an absorbed rabbit anti-D-BSCE serum. The contents of five fractions with SCP activity were combined and concentrated. Each fraction was then subjected to polyacrylamide gel electrophoresis at pH 8.9. The result is shown in Figure 31. It can be seen that at least 2 protein bands were observed in the gels indicating that the SCP in the DTF is contaiminated with one impurity. Work is in progress to obtain the peptide form of SCP in the pure state.

BIOLOGICAL ASSAY FOR THE ANTI-ENCEPHALITOGENIC ACTIVITY OF SCP

1. Assay of encephalitogenicity of β_1-SCP

It was mentioned earlier, that antibody to SCP was detected in the sera of rabbits which had not developed EAE although they had received disease inducing injections of BSC. To ascertain whether or not the pure β_1-SCP isolated from BSC extracts by CM-52 cellulose chromatography was encephalitogenic, guinea pigs were injected with 0.3 mg of the β_1-SCP emulsified in complete Freund's adjuvant (see METHODS). It will be seen from Table V that none of the guinea pigs developed clinical signs of EAE and their sera had anti-β_1-SCP PHA titres ranging from 254 to 2560 on 24th day after the injection. The histological examination was not done because

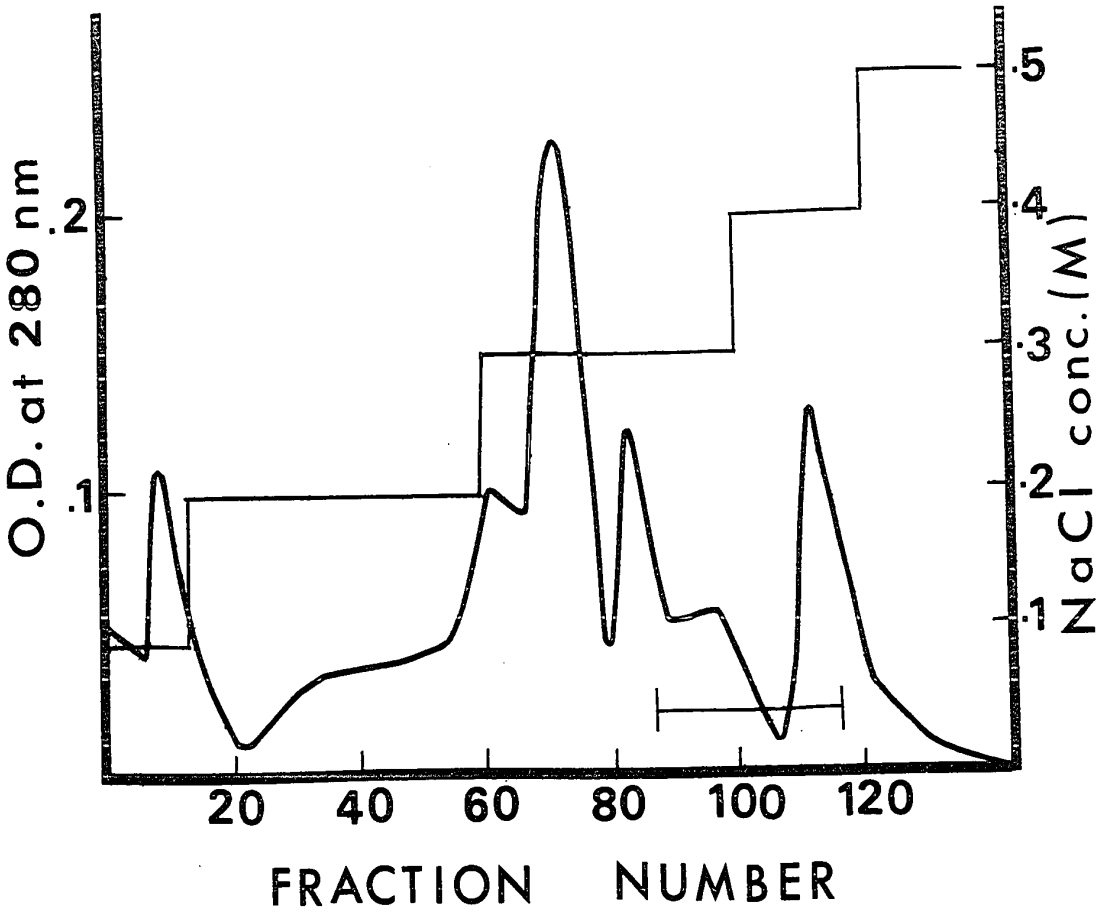

Figure 30. CM-Sephadex C-25 chromatogram of DTF of BSC extracts.

⊢⊣ indicates fractions containing SCP activity.

The proteins were eluted with a stepwise gradient of NaCl in 0.05 M sodium acetate -0.05M NaCl buffer, pH 4.5. The fraction volume was 10 ml and was collected at a rate of 20 minutes per fraction.

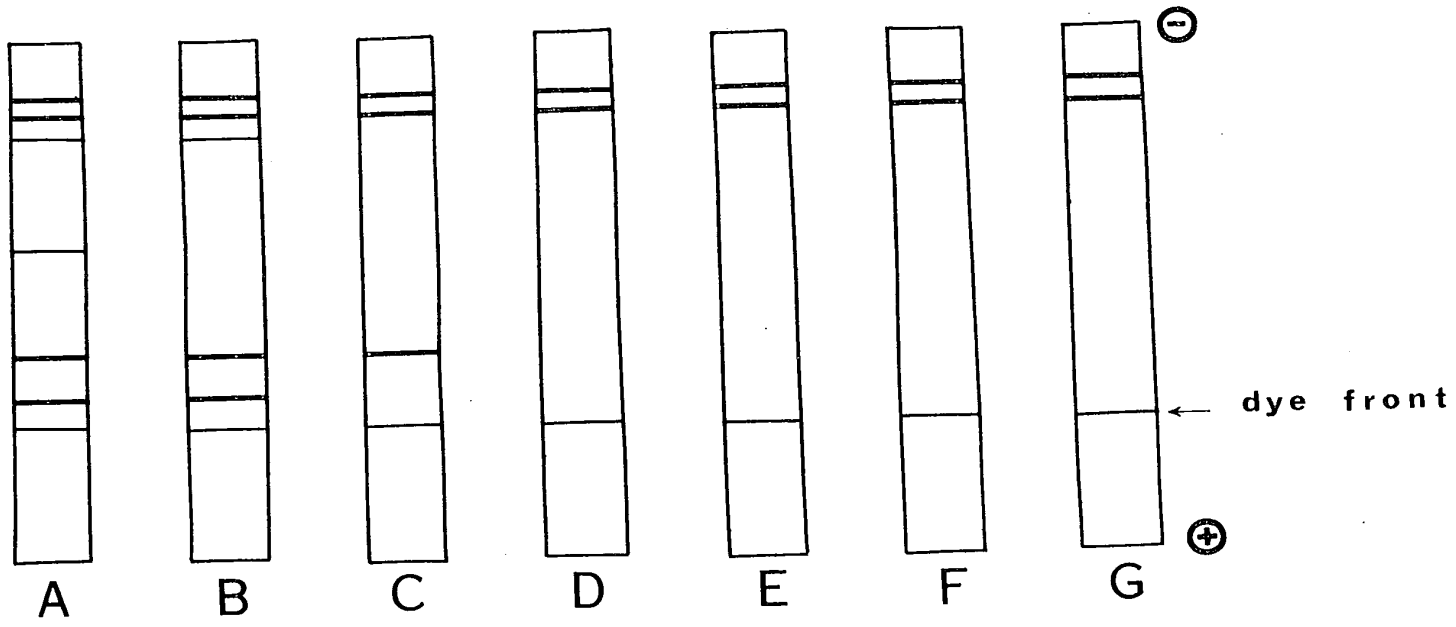

Figure 31. Polyacrylamide gel electrophoretic patterns of fractions 80-90 (A); 91-95 (B); 96-100 (C); 101-105 (D); 106-110 (E); 111-115 (F); 116-120 (G) from chromatography of DTF of BSC extracts on CM-Sephadex C-25 (see Figure 30).

TABLE V

Encephalitogenic activity of β_1-SCP in guinea pigs.

Number of animals	Amount of β_1-SCP injected	Anti-SCP PHA titre at 24 days after injection	Clinical signs: Onset	Clinical signs: Severity	Histologic lesions
6	0.3 mg β_1-SCP in CFA[a]	2/6, 2560	-	-	N.D.[b]
		1/6, 254	-	-	
		1/6, 1,024	-	-	
		1/6, 640	-	-	
		1/6, 1,280	-	-	

a. Each animal was injected with 0.3 ml of an inoculum prepared by emulsifying a solution containing 0.3 mg β_1-SCP with an equal volume of complete Freund's adjuvant containing 4.5 mg of Mycobacterium butyricum per ml.

b. Not done. These animals were kept and hyperimmunized for the production of anti-β_1-SCP serum.

these guinea pigs were kept and reimmunized in order to accumulate a large pool of anti-SCP serum for other experiments.

2. Titration of encephalitogenicity of BSC

EAE was induced in guinea pigs by the injection of bovine spinal cord in complete Freund's adjuvant as described under "Methods". It will be seen from Table VI that 15 mg of BSC contained sufficient encephalitogen to produce severe disease with onset at 14 days in 4 out of 8 animals and somewhat less severe disease with later onset in 3 out of the 4 animals remaining in this group. Thus, 15 mg of BSC is considered a maximal challenge dose for our experimental conditions. Larger doses of BSC induced earlier onset of EAE symptoms (usually by the 11^{th} day after challenge) but did not otherwise influence the severity or morbidity of the disease. The 100 mg dose of BSC is considered an excessive challenge.

Sera were obtained from the animals by cardiac puncture at least once during the course of the experiment. The titre of anti-SCP antibody was determined by the passive haemagglutination (PHA) test as described under "Methods". It can be seen from Table VI that the sera of animals that survived for three weeks after challenge with BSC often contained low levels of anti-SCP antibody which appeared to be associated only with a delay in the eventual development of paralysis. In this connection, it was of interest to note that the serum of one animal which remained healthy and gained weight throughout the experiment had an anti-SCP PHA titre of 16 about 2 weeks after challenge and 512 at the termination of the experiment.

TABLE VI

Encephalitogenicity of bovine spinal cord (BSC) in guinea pigs.

Number of animals	Amount of BSC injected[d]	Clinical signs[a]			Anti-SCP PHA[b] titre		Histologic lesions[c]
		Onset		Severity	on days 13-17	at sacrifice	Severity
	mg		day				
8	15	4/8,	14	+++			++
		3/8,	18-21	++			++
		1/8,	-	-			+
12	30	10/12,	12	+++		0-8(15-18)[e]	++
		1/12,	13	+	0	0 (28)	+
		1/12,	-	-	16	512 (28)	0
6	100	4/6,	12	+++		0 (15-23)	++
		1/6,	12	++		64 (24)	+
		1/6,	15	+	8		+

a. Clinical signs were scored on a cumulative basis as follows: -, no signs; +, weakness and progressive loss of weight; ++, incontinence, transient or partial paralysis of hind legs; +++, paralysis of both hind legs.

b. Passive hemagglutination (PHA) titre was measured with purified β_1-SCP coupled to sheep red blood cells with bis-diazotized benzidine according to (133). The titre is expressed as the reciprocal of the highest dilution of serum that gave a positive pattern.

c. Lesions scored from 0 to ++ acording to (142).

d. Each animal was challenged with 0.3 ml of an inoculum prepared by emulsifying the appropriate amount (wet weight) of BSC in 0.15 ml of saline with 0.15 ml of complete Freund's adjuvant (CFA) containing 4.5 mg of Mycobacterium butyricum per ml.

e. Figures within brackets indicate the number of days between challenge and sacrifice.

3. Pretreatment of guinea pigs with β_1-SCP

To ascertain whether or not the prevention of EAE was mediated by β_1-SCP, 6 guinea pigs each received three weekly injections of 0.1 mg β_1-SCP in incomplete Freund's adjuvant prior to administration of the disease inducing inoculum. This schedule was chosen to ensure that anti-SCP antibody would be present in the sera of the animals before sensitization. The result of the immunodiffusion analyses for anti-β_1-SCP antibody in the sera from 2 guinea pigs is presented in Figure 32. It was noticed that as in the case of guinea pigs injected with β_1-SCP and complete Freund's adjuvant, guinea pigs pretreated with β_1-SCP and incomplete Freund's adjuvant produced good levels of anti-β_1-SCP antibody about one month after receiving the first inoculation of the protein.

4. Antigen-induced prevention of EAE

After it was determined that the guinea pigs pretreated with β_1-SCP responded with the production of suitable levels of anti-β_1-SCP antibody, they were challenged with BSC in complete Freund's adjuvant. The results obtained by pretreatment with β_1-SCP, saline, and proteins prepared from bovine liver and the gray matter of bovine brain are recorded in Table VII. It will be seen that none of the animals which had been immunized with β_1-SCP before challenge exhibited clinical signs of EAE except one guinea pig which lost weight on the 25th day after challenge but gained weight steadily thereafter. No histological lesions were found in the brains of four of these animals

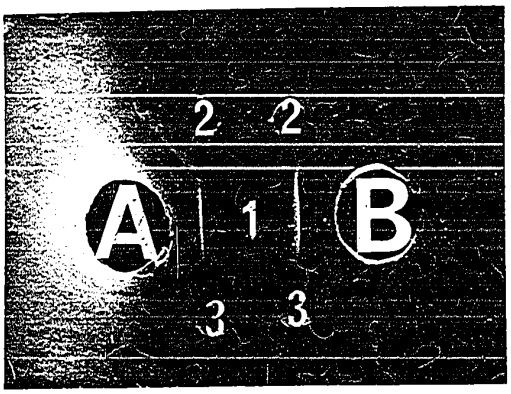

Figure 32. Immunodiffusion analyses of sera from 2 guinea pigs injected with β_1-SCP

(A) Guinea pig # 14.

(B) Guinea pig # 33.

(1) β_1-SCP (0.2 mg protein per ml).

(2) β_1-SCP (0.1 mg protein per ml).

(3) β_1-SCP (0.05 mg protein per ml).

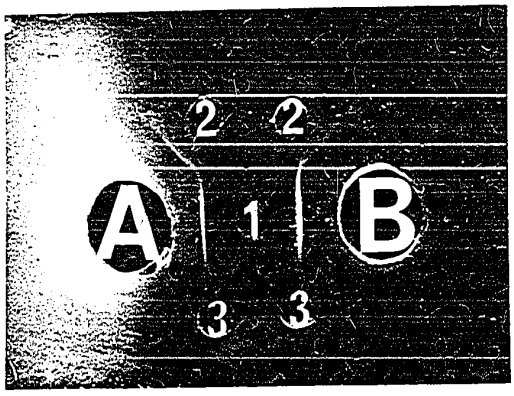

Figure 32. Immunodiffusion analyses of sera from 2 guinea pigs injected with β_1-SCP

(A) Guinea pig # 14.

(B) Guinea pig # 33.

(1) β_1-SCP (0.2 mg protein per ml).

(2) β_1-SCP (0.1 mg protein per ml).

(3) β_1-SCP (0.05 mg protein per ml).

TABLE VII

Prevention of EAE by pretreatment of guinea pigs with β_1-SCP, saline, and proteins from bovine brain and liver.

No. in group	Treatment before Challenge	Induction of EAE	Anti-SCP PHA titre at 14-21 days after Challenge	Anti-SCP PHA titre at Sacrifice	Clinical signs: Onset day	Clinical signs: Severity	Histological lesions: Severity
6	3 weekly injections of saline in IFA[a]	100 mg of BSC in CFA		1/6, neg[b] 2/6, 16 1/6, 32 1/6, 64	4/6, 11 1/6, 17	+++ ++	5/6, ++ 1/6, 0
6	3 weekly injections of 0.1 mg of β_1-SCP in IFA	as above	5/6, 640-2560 1/6, 5120	1280-2560 2560	1/6, 25[c] 5/6, -	- -	4/6, 0 2/6, +
6	3 weekly injections of 5 mg protein of bovine liver in IFA	as above	6/6[d] 0	0-32	4/6, 11 1/6, 13	+++ ++	4/6, ++ 1/6, +
6	3 weekly injections of 5 mg protein of bovine gray matter in IFA	as above	6/6 16-32[e]	8-128	5/6, 11 1/6, -	+++ -	5/6, ++ 1/6, 0

a. Incomplete Freund's adjuvant
b. One animal died before challenge
c. Loss of weight only on 25th day after challenge
d. One animal died on day of challenge
e. Two pigs that survived 3 weeks after challenge

but a few small lesions, graded as 1+ by the criteria of Levine et al. (142), were detected in the brains of two.

In contrast, clinical signs of EAE were shown by five out of six of the guinea pigs that had been pretreated with saline; by five out of six of the guinea pigs that had been pretreated with the protein of bovine brain gray matter; by all of the animals that had been pretreated with the protein of bovine liver. The sera of the animals that had been immunized with the proteins of brain gray matter did not contain enough anti-SCP antibody on the day of challenge to be detected by the passive haemagglutination method. This finding agrees with our results of immunodiffusion analyses carried out earlier which indicated that SCP is not present in the gray matter of bovine brain.

5. Encephalitogenicity of bovine basic protein

Bovine basic protein, provided by Dr. Marian Kies of National Institutes of Health, was titrated for encephalitogenicity under our experimental conditions at 10 μg and 20 μg levels. The results are recorded in Table VIII. It can be seen that 10 μg of basic protein was a barely maximum challenge dose. Of the 5 animals, two had no clinical signs of EAE when the experiment terminated. The rest of the three guinea pigs developed clinical signs of EAE between the second and third week after challenge. The sera did not contain anti-SCP antibody when tested by PHA technique. When the bovine encephalitogenic protein was tested at the 20 μg level, it was found to be more effective in inducing EAE. Of 7 animals tested, 5 showed severe clinical signs after 2 weeks of challenge. Therefore, this dose was used in later experiments.

TABLE VIII

Encephalitogenicity of bovine encephalitogenic protein (BEP) in guinea pigs

Number of animals	Amount of[a] BEP injected µg	Clinical signs[b] Onset	Clinical signs[b] Severity	Anti-SCP PHA titre[c] on days 13-17	Anti-SCP PHA titre[c] at sacrifice	Histologic[d] lesions Severity
		day				
6	10	2/6[e]	12 ++	0	0	++
		1/6	17 ++	0	0	++
		2/6	- -	0	0	0
		3/7	13 +++	0	0	++
7	20	1/7	15 +++	0	0	++
		1/7	18 ++	0	0	++
		2/7	- -	0	0	0-+

a. Each animal was challenged with 0.3 ml of an inoculum prepared by emulsifying the appropriate amount of BEP in 0.15 ml of saline with 0.15 ml of complete Freund's adjuvant (CFA) containing 4.5 mg of Mycobacterium butyricum per ml.

b. Clinical signs were scored on a cumulative basis as follows; - no signs; +, weakness and progressive loss of weight; ++, incontinence, transient or partial paralysis of hind legs; +++, paralysis of both hind legs.

c. Passive hemagglutination (PHA) titre was measured with purified β_1-SCP coupled to sheep red blood cells with bis-diazotized benzidine according to (133). The titre is expressed as the reciprocal of the highest dilution of serum that gave a postive pattern.

d. Lesions scored from 0 to ++ according to (142).

e. One animal died on 5th day after challenge.

6. Prevention of EAE by γ-SCP

When pure γ-SCP became available, it was tested for its ability to prevent EAE. A group of 6 animals were pretreated with γ-SCP in incomplete Freund's adjuvant as described above for β_1-SCP. Seven days after the last injection, they were challenged with 100 μg BEP emulsified in complete Freund's adjuvant. This dosage, which was five times the maximal challenge dose, was chosen so as to ensure that most guinea pigs that received no pretreatment would develop EAE within 2 weeks after challenge. The results are recorded in Table IX. It can be seen that the sera of all of the guinea pigs had anti-SCP PHA titres ranging from 2560 to 5120 before challenge with basic protein. Two weeks after the inoculation of basic protein, anti-SCP PHA titres of the sera dropped to 640 to 1280. It may also be noted from Table IX that none of the guinea pigs developed severe clinical disease, such as paralysis of the hind legs, although 3 of the animals experienced transient loss of weight. Mild histological lesions were found in four of the animals. The experiment shows that pretreatment of guinea pigs with γ-SCP prevented animals from developing EAE when challenged with excessive doses of the encephalitogenic basic protein.

7. Passive administration of antiserum

A pool of anti-β_1-SCP antiserum was raised in guinea pigs as described under "Methods". Figure 33 shows the immunodiffusion analysis of the pooled guinea pig anti-β_1-SCP serum. It can be seen that no precipitin line was observed opposite the basic protein. This confirms our previous observation that SCP does not contain

TABLE IX

Prevention of EAE by pretreatment of guinea pigs with γ-SCP

No. of animals	Treatment before Challenge	Induction of EAE	Anti-SCP PHA titre at			Clinical signs		Histological lesions
			Before Challenge	16-21 days after challenge	Sacrifice	Onset day	Severity	Severity
6	4 injections of 0.1 mg of γ-SCP in IFA[a]	100 µg of BEP in CFA	2/6,2560 4/6,5120	3/6[c],1280 2/6,640	4/6,640 1/6,320	2/6 3/6[b]	- +	2/6, 0 4/6, +

a. Incomplete Freund's adjuvant
b. Loss of weight between 15-17th day after challenge; weight gained steadily afterwards.
c. One animal died 2 days after challenge.

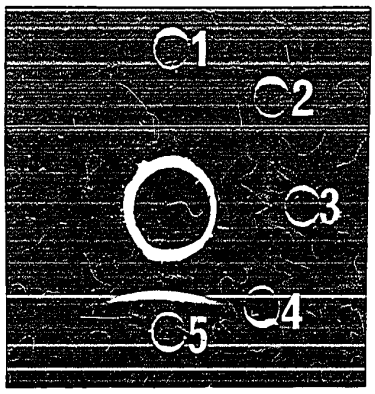

Figure 33. Immunodiffusion analysis of a pooled guinea pig serum containing anti-β_1-SCP antibody.

Peripheral wells

(1) β_1-SCP at a concentration of 0.05 mg protein per ml.

(2) β_1-SCP at a concentration of 0.1 mg protein per ml.

(3) β_1-SCP at a concentration of 0.2 mg protein per ml.

(4) Bovine basic protein at a concentration of 1 mg protein per ml.

(5) DEAE Sephadex A-50 absorbed BSC extracts at a concentration of 1 mg protein per ml.

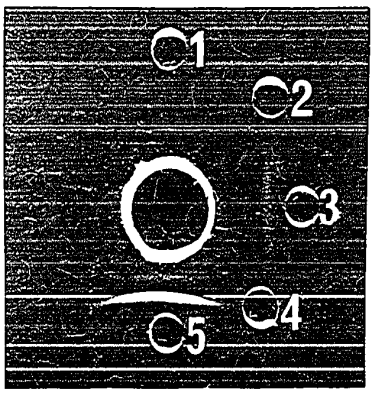

Figure 33. Immunodiffusion analysis of a pooled guinea pig serum containing anti-β_1-SCP antibody.

Peripheral wells

(1) β_1-SCP at a concentration of 0.05 mg protein per ml.

(2) β_1-SCP at a concentration of 0.1 mg protein per ml.

(3) β_1-SCP at a concentration of 0.2 mg protein per ml.

(4) Bovine basic protein at a concentration of 1 mg protein per ml.

(5) DEAE Sephadex A-50 absorbed BSC extracts at a concentration of 1 mg protein per ml.

the same antigenic groups as BEP. The pooled antiserum was found to have an anti-β_1-SCP PHA titre of 5120.

Preliminary studies showed that the antiserum was diluted 40 times when administered passively by intraperitoneal injection. The anti-β_1-SCP PHA titre of serum samples withdrawn at various times from guinea pigs injected intraperitoneally with anti-β_1-SCP serum is shown in Table X. It can be seen that all the antibody had entered the circulation of the recipient 4 hours after it was administered intraperitoneally. The titre remained constant for 12 days, then declined steadily and fell to 8 by 33 days.

8. Passive Protective Experiment

Guinea pigs were injected intraperitoneally with 4 ml of anti-β_1-SCP serum, having a PHA titre of 5120, 4 hours before challenge with 20 µg BEP in complete Freund's adjuvant. This volume was chosen in the hope of obtaining an anti-β_1-SCP PHA titre of 256 in the recipient, calculated on the basis that the antiserum was diluted 40 times when administered passively. Control animals received 4 ml of normal guinea pig serum 4 hours before challenge. The result of the passive protective experiment is shown in Table XI. All the control animals had clinical signs of EAE by 12-15th day after challenge. In the group of animals injected with anti-β_1-SCP serum, only one animal remained healthy till the end of the experiment, while the rest developed EAE by 12-18th day after challenge. This experiment shows that an anti-β_1-SCP PHA titre of 256 at this stage is not enough to protect the animal from the disease.

TABLE X

Anti-SCP PHA titre of serum removed at various times from normal guinea pigs following intraperitoneal injection of 2 ml of anti-β_1-SCP antiserum, PHA titre, 5120.

Number of animals	Treatment	Time after injection of antiserum	Anti-β_1-SCP* (PHA titre)
3	2 ml anti-β_1-SCP antiserum intraperitoneally (PHA titre 5120)	4 hrs.	128
		24 hrs.	128
		3 days	128
		8 days	128
		12 days	128
		14 days	64
		23 days	16
		33 days	8

* Average value of three guinea pigs.

TABLE XI

Prevention of EAE in guinea pigs by passive transfer of guinea pig anti-β_1-SCP antiserum

Number of animals	Treatment before Challenge	Induction of EAE	Anti-SCP (PHA titre)			Clinical signs		Histological lesions
			2 days after injections of antiserum	11th day after challenge	Sacrifice	Onset day	Severity	Severity
6	4cc normal guinea pig serum i.p. 4 hours before challenge	30 mg BSC in CFA		6/6 neg	3/6,neg 1/6,16 1/6,8 1/6,2	4/6,12 2/6,15	+++ +++	++ ++
10	4 cc anti-β_1-SCP serum i.p. 4 hrs. before challenge Subsequent injections of 2cc anti-β_1-SCP antiserum at 3,7,10,12,14,17, and 20 days after challenge	20 µg BEP in CFA	6/10,256 1/10,128 1/10,64 2/10,neg			2/10,12 4/10,15 2/10,17 2/6[a],-	+++ +++ +++ -	++ ++ ++ 0

a. One guinea pig was killed accidently during cardiac puncture on the 6th day after challenge.

IV. DISCUSSION

A. ANTIGENS IN BOVINE SPINAL CORD EXTRACTS

The specificity of a protein antigen is altered when the protein is denatured by heat, or by strong acids or bases. Although denatured proteins are also antigenic, the antibodies formed in response to the administration of denatured protein are different from those directed against the native protein. Several investigators (143,4,5) have used saline rather than acids or bases to extract brain proteins so as to avoid denaturation of the proteins.

In the present study, it was noted that the SCP in the undefatted bovine spinal cord could be extracted with 0.1 M sodium chloride. If the residue that remained after several washings with 0.1 M sodium chloride was defatted with chloroform-methanol (2:1), only trace amounts of SCP were obtained when the residue was subsequently extracted with sodium acetate buffer, pH 4.3. When the nuclear, mitochondrial, microsomal, and soluble fractions of fresh bovine spinal cord were separated by differential centrifugation, only the soluble fraction formed a precipitin band with anti-BSC serum in immunodiffusion analyses. The encephalitogenic basic protein has been proved to be a constituent of the myelin. However, unless lipid is removed from the myelin, it is extremely difficult to isolate this basic protein even with strong acids (144). The location of SCP in the soluble fraction of bovine spinal cord homogenates also indicates that SCP is probably not a myelin protein.

SCP has been found to be one of the major immunogens of BSC that induces humoral antibodies. Rabbits that did not develop EAE when injected with whole BSC in

complete Freund's adjuvant had precipitating anti-SCP antibodies in their sera within a month after challenge. Antibodies to SCP could also be elicited in rabbits with one injection of the partially purified BSC extracts (absorbed with DEAE-Sephadex A-50) or the purified forms of SCP.

It has been observed that most nervous tissue proteins are poor immunogens (145). Thus, Hatcher (146) reported that it took 6 months for rabbits injected with bovine brain extracts in complete Freund's adjuvant to produce suitable working levels of precipitating anti-brain antibodies. Liakopoulou (147) observed that rabbits which received one injection of rat brain extracts emulsified in complete Freund's adjuvant produced only low concentrations of antibodies. The weak immune response generated by these antigens may have been due to their low concentration in brain extracts or may be due to the generally weak immunogenic properties of brain proteins.

Double diffusion analyses of BSC extracts developed with an absorbed rabbit anti-BSC or anti-D-BSCE serum demonstrated the presence of five to seven antigens. However, in BSC extracts, most of the antibodies were directed against a particular protein of spinal cord. It can be seen in Figure 5 that most of the antigen-antibody precipitin bands did not form when diluted solutions (1.0 mg protein per ml) of BSC extracts were used although bands were formed by SCP even at concentrations as low as 0.1 mg protein per ml of the BSC extracts.

Immunoelectrophoretic analyses of the BSC extracts revealed that the SCP existed in two molecular forms as shown by the formation of a double arc. One form has the

electrophoretic mobility of a β_1-globulin and the other the electrophoretic mobility of a γ-globulin. In this respect, it is of interest to note that the main SRANT (species-restricted antigen of nervous tissue) protein of rat brain extracts (147) also exists in two molecular forms having the electrophoretic mobilities of a α_1-globulin and an albumin respectively. The existence of a protein in multiple forms within an organ could mean that each form has its own specific function in maintaining the integrity of the organ.

When the material in the dialysis tubing filtrate (DTF) was analyzed by the immunodiffusion technique, it was found to contain a peptide which is immunologically identical to the two forms of SCP retained by the dialysis tubing (Figure 8). The SCP peptide that passes through dialysis tubing is found in extracts of spinal cord whether or not BSC is first defatted with chloroform-methanol, which inhibits brain proteases (140). Homogenization of BSC in the presence of cupric ion, which also inhibits brain proteases did not influence the amount of SCP that passed through the dialysis tubing. Thus, it is probably not a breakdown product of the larger forms of SCP, due to neutral brain protease activity (141). In this connection, it is of interest to note that the γ_c-globulin of bovine CSF and human CSF also occurs in multiple molecular forms having different surface charges and different molecular sizes (23, 148, 149). In these cases too, the small form that passes through dialysis tubing also has the electrophoretic mobility of a γ-globulin.

To investigate the general chemical nature of the SCP, BSC extracts were heated at 100°C for 30 minutes, or dialyzed against glycine-HCl buffer, pH 2.0; Na-acetate-acetic acid buffer, pH 4.0; Tris-HCl buffers, pH 6 and 8; and Na-borate-NaOH buffer,

pH 10.0. None of these treatments destroyed the antigenic activity of the SCP indicating that it is a stable protein. The encephalitogenic basic protein is also remarkably stable for its biological activity is not destroyed by autoclaving or by exposure to pH 2.5 or pH 11.6.

Immunodiffusion analyses of human, rabbit, rat and mouse spinal cord extracts indicated that the SCP from these mammalian species were immunologically (but not quantitatively) identical with the bovine SCP (Figure 11). The mouse spinal cord extracts, even at a concentration of 11 mg protein per ml showed a much weaker reaction than the spinal cord extracts of other species. This would suggest either that the SCP content of mouse spinal cord extracts is relatively low or that the non-antigenic portions of the SCP molecule in the mouse may differ considerably from the non-antigenic part of bovine SCP. The rat SCP also occurs in two molecular forms having different electrophoretic mobilities (Figure 14).

Bovine spinal cord extracts were found to contain several antigens which were shared with other bovine organs such as the liver, and the adrenal. However, when such bovine organ extracts were analyzed by immunodiffusion experiments with a monospecific rabbit anti-SCP serum, no precipitin band corresponding to the band formed by SCP was observed (Figure 13). This indicates that SCP is a tissue specific antigen which is restricted to the central and peripheral systems. It is interesting to note that SCP was present in the saline extracts of bovine peripheral nerve in roughly the same concentration as in bovine spinal cord extracts. The SCP in peripheral nerve extracts also occurs in two

molecular forms as shown in Figure 14.

Bovine encephalitogenic basic protein did not react in immunodiffusion analyses with a monospecific rabbit anti-SCP serum indicating that SCP and BEP are two entirely different proteins and do not share any antigenic determinants.

The immune response to an organ specific antigen has been attributed to be the main pathogenetic factor in the development of autoimmune diseases such as EAE, orchitis, and thyroiditis (150). In the present study, the rabbits were repeatedly immunized with partially purified BSC extracts or purified forms of SCP for over 2 years and remained healthy. Yet is can be seen from Figure 11 that the animals' sera contained antibody directed against an organ-specific antigen of their nervous tissues.

Recent studies on protein synthesis during the maturation of the brain have shown that the protein content increases in all areas of the maturing brain (151, 152), and that the amino acid composition of brain proteins also differs in young and adult animals. Studies on myelination also shows that the "early" myelin formed during the first few weeks of myelination had a different composition from that of mature myelin (153). In the case of kitten optic nerve, myelination begins about 10 days after birth. Agrawal et al. (154) have isolated a myelin-like fraction from developing brain and found it to be devoid of basic protein. Only relatively small amounts of this fraction can be isolated from the adult brain. In the present study, it was observed that the spinal cord extracts obtained from newly born, 5-day-old and 8-day-old rats were found to be devoid of SCP (Figure 17). SCP was first detected in spinal cord extracts from 9-day-old rats. One can argue that the spinal cord extracts from newly born rats were not concentrated enough to

allow detection of SCP. Due to the limited amount of neonatal spinal cord, it was not possible to concentrate the spinal cord extracts from newly born or 5-day-old rats. However, even if they had been concentrated to 20 mg protein per ml, it is doubtful if SCP would have been detected, since no SCP was found in spinal cord extracts from 8-day-old rats concentrated to 14 mg protein per ml. Spinal cord extracts of 9-day-old rats contained a low level of SCP (Figure 17). An absorbed rabbit anti-BSC serum was used for these immunodiffusion analyses. As shown in Figure 11, 4 times as much rat spinal cord protein (compared with BSC protein) was needed in order to detect SCP activity. Obviously, the determination of the time at which SCP appears first during the development of the rat nervous system would be more accurate if an antiserum directed against rat spinal cord extract were available.

The BSC extracts were first partially purified by batch-wise absorption with DEAE-Sephadex A-50 at pH 7.0. This treatment removed all the acidic proteins present in the extracts (Figure 23,2) but left the SCP activity.

The partially purified BSC extracts were resolved into five fractions by chromatography on CM-52 cellulose. Pure SCP with the electrophoretic mobility of a β_1-globulin was obtained from the ascending limb of peak 5 (Figure 19). The SCP peptide was found to be eluted at the beginning of the chromatogram, when 0.05 M sodium chloride was used as eluant. When a concentrated solution of DTF, which contains only the peptide form of SCP, was applied to a CM-52 cellulose column under similar conditions, the SCP peptide was detected in the fraction eluted by 0.05 M sodium chloride. Sober and

Peterson (155) stated that chromatography of a protein on cellulose ion-exchangers is dependent on several factors such as the surface charge, size and a specific affinity of a non-electrostatic nature between the adsorbent and the protein. As far as size is concerned, a protein with a low molecular weight would be expected to move down the column faster than a protein with the same surface net charge but a higher molecular weight, since the smaller protein is able to form fewer bonds with the adsorbent. Thus, Lospalluto (156) noted that a fraction appearing at the end of a chromatogram of serum on DEAE-cellulose was a 19S γ-globulin. One would expect it to be eluted right after γG if chromatography of proteins on ion-exchange cellulose was based solely on net surface charges. Similarly, Eylar et al. (41) had noted that basic proteins were eluted in order of increasing sizes when chromatographed on a CM-cellulose column.

The purified form of SCP with an electrophoretic mobility of a β_1-globulin was shown to yield only one band after it was subjected to electrophoresis in 7% polyacrylamide gel at alkaline pH (Figure 23,3). It formed only one precipitin line when it was allowed to diffuse against an unabsorbed rabbit anti-BSC serum (Figure 25).

The molecular weights of the three forms of SCP were determined by exclusion gel chromatography on a calibrated Sephadex G-100 column. The SCP forms retained by dialyzing tubing and having β_1-and γ-globulin electrophoretic mobilities were found to have molecular weights of 18,000 and 12,000 respectively; the peptide form of the antigen in the DTF had a molecular weight of 4,500. A concentrated solution of DTF was used for the estimation of molecular weight of the dialysable form of SCP. The tube

in which the highest SCP activity was found by immunodiffusion analyses was taken as the effluent volume for calculating the molecular weight of SCP in the DTF. The molecular weight of the dialyzable form of SCP determined in this way is not as accurate as it would be if pure SCP peptide had been available.

The molecular weight of the SCP having a β_1-globulin electrophoretic mobility was also determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and was found to be 19,500. It has been shown that the electrophoretic mobility of a protein in the presence of SDS is a function only of the size of the polypeptide chain. Thus, all proteins migrate as anions as the result of complex formation with SDS, which is capable of disrupting hydrogen, disulphide and other bonds in a protein molecule. If the β_1-SCP were composed of subunits linked by disulphide bonds, electrophoresis in the presence of SDS would have revealed more than one band. However, only one band was observed, indicating that the purified β_1-SCP was electrophoretically homogeneous and composed of a single polypeptide chain. The molecular weight of β_1-SCP determined by this method was in good agreement with that determined by gel filtration.

The amino acid composition of β_1-SCP shows that it contained 21% dicarboxylic acid and 16% basic amino acids. Tryptophan was not determined and no half cystine was present indicating that no disulphide linkage exists in the β_1-SCP molecule.

Further studies will have to be done to establish how the three molecular forms of SCP are related structurally. For the present, however, it is interesting to note that the estimated molecular weights of the different forms are multiples of 6,000. Thus, the

three forms might be respectively a monomer, a dimer and a trimer of a polypeptide chain. This speculation is strengthened by the fact that when the three forms of SCP react with anti-BSC serum in immunodiffusion experiments, the precipitin lines opposite each fuse with no trace of spur formation. It is difficult to understand how two molecules one of which is 1/3 to 1/4 the size of the other could form immunologically identical precipitin bands that fuse unless the larger one is composed of repeating units of the small one. The immunodiffusion studies indicate that the smallest molecular form of SCP contains all the antigenic sites of the larger forms. The structural relationships of the three forms may be clarified once the amino acid composition of the γ-SCP and the SCP peptide are determined.

The exact location of SCP in the white matter of the spinal cord has not been established. The direct fluorescein tagged antibody technique has been applied to fresh bovine brain and spinal cord tissues without success. As mentioned earlier, SCP is not a firmly bound tissue antigen and it may diffuse from the tissue during the stage where washing with buffered electrolyte solutions is required. Preliminary studies show that treatment of nervous tissues with glutaraldehyde, alcohol or isopentane had a deleterious effect on the antigenic reactivity of the SCP. In order that the fluorescent antibody technique may be applied, other methods of fixing this soluble antigen in situ will have to be investigated.

B. THE BIOLOGICAL PROPERTY OF SCP

The discovery that anti-SCP antibody was the most prominent precipitin in the

sera of rabbits that did not develop EAE after they received a disease inducing injection of BSC in complete Freund's adjuvant prompted us to investigate the capacity of anti-SCP antibody to prevent the development of EAE. EAE can be prevented by (1) injection of encephalitogenic basic protein alone or emulsified with incomplete Freund's adjuvant, or (2) by injection of complete Freund's adjuvant alone. So far, only Einstein et al. (109) reported several years ago that EAE could be suppressed in guinea pigs by the injection of a non-encephalitogenic basic protein isolated from the brain. The exact mechanism by which disease was suppressed is not clear as no investigations were carried out to determine whether or not humoral antibodies were produced.

The experiments in the present study were carried out with guinea pigs because the incidence of induction of EAE with a maximal dose of bovine spinal cord is known to approximate 80% in this species whereas the incidence of induction of the disease in rabbits is neither as high nor as predictable. Purified β_1-SCP was found to be non-encephalitogenic in guinea pigs. Doses up to 300 µg of SCP emulsified in complete Freund's adjuvant were injected and the animals remained healthy throughout the experiment.

Preliminary orienting experiments had indicated that the sera of guinea pigs that had been injected three times at weekly intervals with 0.1 mg of β_1-SCP in IFA had anti-SCP PHA titres ranging from 512 to 2560. Usually, the antibody levels were high enough to support immunodiffusion tests (Figure 32). It has been assumed that the animals pretreated with SCP before challenge responded in similar fashion but their sera were not always monitored at the time of challenge because of the risks inherent in withdrawing

blood by cardiac puncture. It will be noted from Table VII that two weeks after challenge with BSC, the sera of the guinea pigs pretreated with β_1-SCP had PHA titres ranging from 640 to 5120, and that the titres were substantially unchanged at the termination of the experiment. Guinea pigs which were pretreated with γ-SCP in IFA had PHA titres ranging from 2560-5120 before challenge. The sera from these guinea pigs had PHA titres ranging from 640 to 1280 two weeks after challenge (Table IX), and the PHA titres had decreased to the 320-640 range at the termination of the experiment.

The PHA titre at two weeks after challenge was about one-half the titre at the time of challenge. The average half-life of antibody has been shown to be about two weeks (157). In this experiment, the animals did not receive any SCP after the final pretreatment injection one week before challenge and so the decline in antibody titre would begin earlier than it would have if the guinea pigs had been challenged with 100 mg BSC which has been calculated to contain about 0.05 mg SCP. The PHA titres at two weeks after challenge and at the termination of the experiment were similar in guinea pigs pretreated with β_1-SCP. Thus, animals challenged with BSC were being stimulated with SCP at the same time. This may account for the unchanged PHA titres at the termination of the experiment. An excessive dose of BSC was used for challenge in the present studies in order to conduct a rigorous test of the protective capacity of β_1-SCP.

Our results suggest that a critical level of anti-SCP antibody must be present in order to prevent the development of EAE. For example, animals whose sera had PHA titres less than 64 developed clinical EAE although the onset of the symptoms was delayed.

It will also be noted that one animal that was challenged with 30 mg of BSC but remained healthy had anti-β_1-SCP titres of 16 and 512 at two and four weeks after challenge. It would appear that the critical anti-β_1-SCP titre is higher than 64 but less than 512.

It will be seen from Table VII that pretreatment of guinea pigs with bovine liver protein and bovine brain gray matter protein before challenge with BSC did not prevent them from developing EAE. The anti-β_1-SCP PHA titres in their sera ranged from 0-64.

Although pretreatment of guinea pigs with SCP before sensitization with either BSC or encephalitogenic basic protein prevented the development of clinical signs of EAE, it did not protect some animals from developing mild histological lesions. Histological lesions were also observed (110, 112) in animals in which clinical EAE had been prevented or suppressed by injections of chemically modified encephalitogenic basic protein. Antigen-induced suppression of EAE has been attributed to desensitization (105), in which the excess antigen interacts with sensitized lymphocytes preventing them from reaching the target in the central nervous system. The presence of mild histological lesions in treated animals suggests that some sensitized lymphocytes were not blocked and were able to reach the target organ. One might speculate that more antigen, in the case of prevention with encephalitogenic protein or higher concentrations of anti-SCP antibody in the case of protection mediated by SCP, are needed in order to prevent sensitized lymphocytes from reaching the central nervous system.

The mechanism of the protective action of SCP is not clear at present but the evidence that we have accumulated to date suggests that the protective effect is mediated

by anti-SCP antibody. As SCP is not a myelin protein, it is unlikely that the protective action of the anti-SCP antibody is immunologically specific for lymphocytes sensitized to the encephalitogen of myelin.

Paterson et al. (103) reported that the administration of serum from rats that had recovered from EAE protected the recipients from developing the disease. Our attempts to confer protection in guinea pigs by passive administration of anti-β_1-SCP serum were not successful. The failure was probably due to the use of insufficient amounts of antibody as the anti-β_1-SCP PHA titres in the sera of the recipients were never higher than 256. This is about 1/10 to 1/20 the antibody titres in sera of actively immunized animals that were protected from EAE by challenging injections of BSC. Additional experiments will be performed with concentrated solutions of anti-SCP IgG.

V. SUMMARY AND CONTRIBUTIONS TO KNOWLEDGE

1) A highly immunogenic spinal cord protein (SCP) has been extracted from bovine spinal cord with 0.1 M sodium chloride. It is located in the soluble fraction of the homogenate and can stimulate high levels of precipitating antibodies in rabbits and guinea pigs.

2) The protein is a nervous tissue antigen that is found mainly in the white matter of the spinal cord in the central nervous system and is apparently absent from gray matter. The antigen was also found in extracts of spinal cords from other mammalian species.

3) The antigen exists in three molecular forms. Two forms are large enough to be retained by dialysis tubing and have electrophoretic mobilities of a β_1-globulin (β_1-SCP) and a γ-globulin (γ-SCP) respectively. The dialysable form of the antigen has an electrophoretic mobility of a γ-globulin. The molecular forms of the antigen retained by dialysis tubing, β_1-SCP and γ-SCP were estimated by gel filtration chromatography to have molecular weights of 18,000 and 12,000 respectively. The SCP peptide had a molecular size approaching 5,000.

4) The antigen was not detected in extracts of rat spinal cord until the 9th day after birth. Thus, the appearance of SCP precedes myelination in this species.

5) Pretreatment of guinea pigs with β_1-SCP or γ-SCP prevented them from developing

experimental allergic encephalomyelitis when they were subsequently challenged with excessive doses of bovine spinal cord or bovine encephalitogenic basic protein.

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